University of Alberta

The Role of Gibberellins and Auxins in Pea Fruit Development

by

Henderika Fransina van Huizen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of doctor of philosophy

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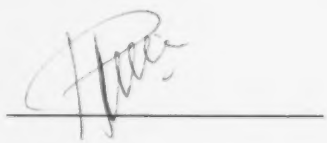

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Abstract

Pea (*Pisum sativum* L.) fruit is a model system for studying the effect of seeds on fruit development, a process that involves cell division, elongation and differentiation. The effect of seeds on pea pericarp growth is assumed to involve hormones. This thesis investigated the role of gibberellins (GAs) and auxin (4-Cl-IAA) and their interaction in pea fruit development. Specifically, this research focused on the investigation of 4-Cl-IAA as a seed-derived regulator of GA biosynthesis in pea pericarp. [^{14}C]GA_{19} metabolism studies confirmed that the conversion of GA_{19} to GA_{20} in pea pericarp is seed regulated and that the auxin 4-Cl-IAA can substitute for the seeds in the stimulation of pericarp growth and the conversion of GA_{19} to GA_{20}. To further understand how seeds and 4-Cl-IAA regulate the conversion of GA_{19} to GA_{20}, GA 20-oxidase gene expression in pea pericarp was investigated. Our results show that GA 20-oxidase mRNA levels in pea pericarp are maintained when seeds are present, but decrease when seeds are removed. Furthermore, mRNA levels of this gene are enhanced by 4-Cl-IAA and reduced by GA_3. The metabolic studies as well as the molecular studies suggest that the conversion of GA_{19} to GA_{20}, is controlled by seeds and 4-Cl-IAA, at least in part, by regulating GA 20-oxidase mRNA levels. To assess at the protein level the role of GA and 4-Cl-IAA in pea fruit development, *in vivo* labeling with [^{35}S]methionine coupled with two-dimensional gel electrophoresis was used to characterize *de novo* synthesis of proteins during GA_3, 4-Cl-IAA, and seed-induced pea pericarp growth. The polypeptide changes could be grouped into 6 classes: 4-Cl-IAA-induced, 4-Cl-IAA-repressed, GA_3-induced, GA_3- and 4-Cl-IAA-induced, GA_3- and 4-Cl-IAA-repressed, and seed-induced. In general, application of GA_3 plus 4-Cl-IAA replaced the seed effects on pericarp protein synthesis. Histone H2A and γ-TIP genes were used as molecular probes to follow the pattern of cell division and enlargement, respectively, during early pea fruit development and in hormone treated pericarp. These

studies as well as histological studies indicated that GA_3 and 4-Cl-IAA are both required for pea pericarp development.

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LIST OF ABBREVIATIONS

ABP	auxin binding protein
ANOVA	analysis of variance
BSA	bovine serum albumin
CPP	copalyl pyrophosphate
cDNA	complementary deoxyribonucleic acid
4-Cl-IAA	4-chloroindole-3-acetic acid
C-19	carbon-19
C-20	carbon-20
CHAPS	3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate
DAA	days after anthesis
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GA	gibberellin
GBP	gibberellin binding protein
GC-MS-SIM	gas chromatography-mass spectrometry-selective ion monitoring
GGPP	geranyl geranyl pyrophosphate
IAA	indole-3-acetic acid
IEF	isoelectric focusing
HPLC	high pressure liquid chromatography
kb	kilobase
kD	kiloDaltons
KRI	Kovats retention index
LD	long day
LSD	least significant difference
mRNA	messenger ribonucleic acid
NAA	naphthaleneacetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PMSF	phenyl methyl sulfonyl fluoride
PVP	polyvinylpyrrolidone

RNA	ribonucleic acid
SDS	lauryl sulfate sodium salt
SE	standard error
SP	split pericarp
SPNS	split pericarp no seeds
SSC	sodium chloride/sodium citrate
SSPE	sodium chloride/sodium phosphate EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TCA	trichloroacetic acid
TIP	tonoplast intrinsic protein
TLC	thin-layer chromatography
v/v	volume per volume

Chapter 1

INTRODUCTION

Pea Fruit Development

Fruit development involves a complex interaction of molecular, biochemical, and structural changes that transform a fertilized ovary into a mature fruit. Fruit set and sustained pericarp elongation in pea are dependent on the presence of fertilized ovules. In order to elucidate the role of seeds in pea pericarp (pod) elongation, it is important to understand the growth patterns and the major developmental stages of the pericarp and the seeds. The pea plant is self-pollinating. Pollination takes place between 24 and 35 h before the open flower stage. Fertilization has occurred by the time the flower is fully opened (Cooper, 1938). The pea fruit contains a single ovary bearing typically between 8 to 10 ovules placed alternately along the ventral suture. All ovules may not be fertilized, or may not continue development (Cooper, 1938). The growth of the pea pericarp is sigmoidal. In general, the pericarp is in the linear phase of growth between 3 and 7 days after anthesis (DAA). From day 8 the growth rate of the pod decreases and elongation ceases at 12 DAA (Eeuwens and Schwabe, 1975).

Growth of seeds follows a double sigmoid curve; two periods of rapid growth are separated by a short lag phase with a slower growth rate (Eeuwens and Schwabe, 1975). Cell expansion in the pericarp tissue is not paralleled by developmental events in the seed. During the linear phase of pericarp growth, seeds contain less than 5 µl of liquid endosperm and the embryo is at its globular stage (0.2-0.6 mm in diameter; Marinos, 1970). The embryo enlarges rapidly after 8 DAA and its growth is correlated with the resorption of the endosperm (Marinos, 1970; Eeuwens and Schwabe, 1975). By the middle of the lag phase of seed growth (around 14 DAA) the embryo has filled up the embryo sac (Eeuwens and Schwabe, 1975). Rapid growth and differentiation of the

embryo occurs during the post-lag phase (16 to 32 DAA). Finally at the mature fruit stage, seeds contribute more than 75% to the total dry weight of the fruit (Eeuwens and Schwabe, 1975).

It is generally accepted that in normal fruit development, the developing embryo controls the rate and sustenance of cell division and cell enlargement of the ovary (Varga and Bruinsma, 1986). If ovules do not develop seeds in part of a fruit, lopsided fruits are formed in which normal and retarded organ development coincide closely with the presence or absence of seeds respectively (Nitsch et al., 1960). In pea fruit, a positive correlation exists between the number of developing seeds and pericarp growth (Ozga et al., 1992). There is currently very little information about the mechanisms by which developing seeds regulate cell division and enlargement of fruit.

Fruit development in pea provides a useful model system to define the signals produced by the developing seeds that direct cellular activities in the surrounding ovary. It is likely that developing seeds produce signal molecules that regulate cell division, cell expansion and sink activity of the surrounding fruit cells (Gillaspy et al., 1993). Such signal molecules could be transported by apoplastic diffusion, through plasmodesmata or vascular connections. The plant growth regulators, auxins and/or gibberellins (GAs), are likely candidates for such signal molecules (Gillaspy et al., 1993).

Parthenocarpy, i.e., the formation of seedless fruits has contributed much information about the role of hormones in early stages of fruit development. Parthenocarpic fruit development can be induced in a range of plant species by treatment with different plant growth substances, which thereby substitute for the presence of fertilized ovules (Nitsch, 1970). In addition, developing fruits and, particularly, immature seeds are rich sources of auxins (Ernstsen and Sandberg, 1986; Katayama et al., 1988), GAs (Hedden et al., 1993; Talon et al., 1992) and cytokinins (van Staden, 1983), and in

some cases, correlations between fruit growth and the levels of endogenous hormones in ovaries have been found (De Bouille et al., 1989; Garcia-Martinez et al., 1991). Although parthenocarpic development of pea fruits may be obtained by applying GAs, auxins or cytokinins to seedless ovaries (Garcia-Martinez and Carbonell, 1980, 1985), only GAs produce fruits with similar shape and size to pollinated fruits.

Eeuwens and Schwabe (1975) observed that destruction of the seeds by needle-pricking 2 days after anthesis (DAA) inhibited pea pericarp elongation and the pericarp subsequently abscised. Application of the synthetic auxin, naphthaleneacetic acid (NAA), or GA_3 could partially restore pericarp elongation. Pericarp elongation could be fully restored by application of a mixture of GA_3 and NAA. To gain a better understanding of the hormone requirements for pea fruit development, Ozga et al. (1992) developed a split pericarp technique in which the pericarp remained attached to the plant. In this system, fruits are split down the dorsal suture, while pericarp and seeds can continue to grow until maturity. Splitting of the pericarp 2 DAA without disturbing the seeds resulted in 20 to 25% reduced pericarp growth compared to the intact pericarp (Ozga et al., 1992). The advantage of this split-pod technique is that it allows the manipulation of seeds while maintaining viable seeds and elongating pericarp tissue. Results obtained using the split-pericarp system showed that GAs as well as the auxin, 4-Cl-IAA, can substitute for the seeds in the stimulation of pericarp growth (Ozga and Reinecke, 1994; Reinecke et al., 1995).

Data from experiments involving seed removal or seed destruction and exogenous GA and auxin applications (Eeuwens and Schwabe, 1975; Ozga et al., 1992) strongly suggest that GAs and auxin (4-Cl-IAA) naturally present in pollinated ovaries early in development (0-6 DAA; Garcia-Martinez et al., 1991; Ozga et al., 1993) regulate pericarp growth.

Biosynthesis of Gibberellins

Gibberellins (GAs) are a large family of diterpenoid compounds, characterized by an *ent*-gibberellane backbone (Takahashi et al., 1990). GAs control many diverse plant processes such as germination, cell elongation and division, and flower and fruit development (reviewed in Davies, 1995; Pharis and King, 1985; Takahashi et al., 1990). Immature seeds, in particular, are rich sources of GAs and have been used in many studies involving quantitation and characterization of endogenous GAs (Khan, 1982; MacMillan, 1984; Pharis and King, 1985). Much knowledge concerning GA biosynthesis and metabolism is derived from studies with immature seeds (Sponsel, 1985) or cell-free extracts from seeds (Kamiya and Graebe, 1983; Graebe, 1987). Gibberellin biosynthesis occurs into three phases: (1) the biosynthesis of *ent*-kaurene, (2) the biosynthesis of GA_{12}-aldehyde and (3) the biosynthesis after GA_{12}-aldehyde.

During the first phase, *ent*-kaurene, which is the first committed intermediate in GA biosynthesis, is formed from mevalonic acid via 10 enzymatic reactions. All of the steps of *ent*-kaurene biosynthesis are common to the biosynthesis of other terpenoids except for the last step, in which geranyl geranyl pyrophosphate (GGPP) undergoes cyclizaton. The cyclization of GGPP to *ent*-kaurene is a two-stage reaction catalysed by the enzymes *ent*-kaurene synthase A and *ent*-kaurene synthase B. *ent*-Kaurene synthase A catalyses the conversion of GGPP to the bicyclic intermediate copalyl pyrophosphate (CPP), whereas *ent*-kaurene synthase B catalyses the further conversion of CPP to the tetracyclic diterpene *ent*-kaurene (Duncan and West, 1981).

In the second phase of GA biosynthesis, *ent*-kaurene undergoes a series of oxidation reactions that result ultimately in the production of GA_{12}-aldehyde (Graebe,

1980). GA_{12}-aldehyde is the first-formed GA in all systems and thus appears to be the precursor of all other GAs.

In the third and final phase of GA biosynthesis, an array of oxidation and hydroxylation reactions convert GA_{12}-aldehyde into other GAs. In pea, the early 13-hydroxylation pathway is active (GA_{12} -> GA_{53} -> GA_{44} -> GA_{19} -> GA_{20} -> GA_1; Fig. 1; Graebe, 1987). GA_1 is considered to be the biologically active endogenous GA in pea internode elongation (Phinney, 1984). It is likely that it has a similar function in pea pericarp. 2ß-hydroxylation of GA_{20} and GA_1 forms GA_{29} and GA_8 respectively (Fig. 1.1), which are biologically inactive GAs in pea internode growth.

Because *ent*-kaurene is a key intermediate in the GA pathway, its synthesis is likely to be a regulatory point for GA biosynthesis. *ent*-Kaurene production has been shown to be altered by changes in photoperiod in spinach (Zeevaart and Gage, 1993), and changes in temperature and growth potential in pea (Chung and Coolbaugh, 1986; Moore and Coolbaugh, 1991).

Recently *ent*-kaurene synthase A has been cloned from *Arabidopsis thaliana* by genomic subtraction (Sun et al., 1992). To obtain the protein cDNA clones encoding *ent*-kaurene synthase A were overexpressed in *Eschericha coli* (Sun and Kamiya, 1994). The N-terminal side of the *ent*-kaurene synthase A protein had features common to transit peptides of many chloroplast proteins. The structure of the protein and data from chloroplast import experiments indicate that *ent*-kaurene synthase A can be translocated into plastids, and it is likely to be a stromal protein (Sun and Kamiya, 1994). Yamaguchi et al., (1996) cloned and characterized a cDNA encoding the *ent*-kaurene synthase B from pumpkin. *ent*-Kaurene is metabolized to GAs by membrane-associated monooxygenases and soluble 2-oxoglutarate-dependent dioxygenases (Graebe, 1987). The latter group includes enzymes responsible for successive oxidations at carbon-20 (C-20), leading to its loss as CO_2 and hence to the formation of the biologically active carbon-19 (C-19) GAs.

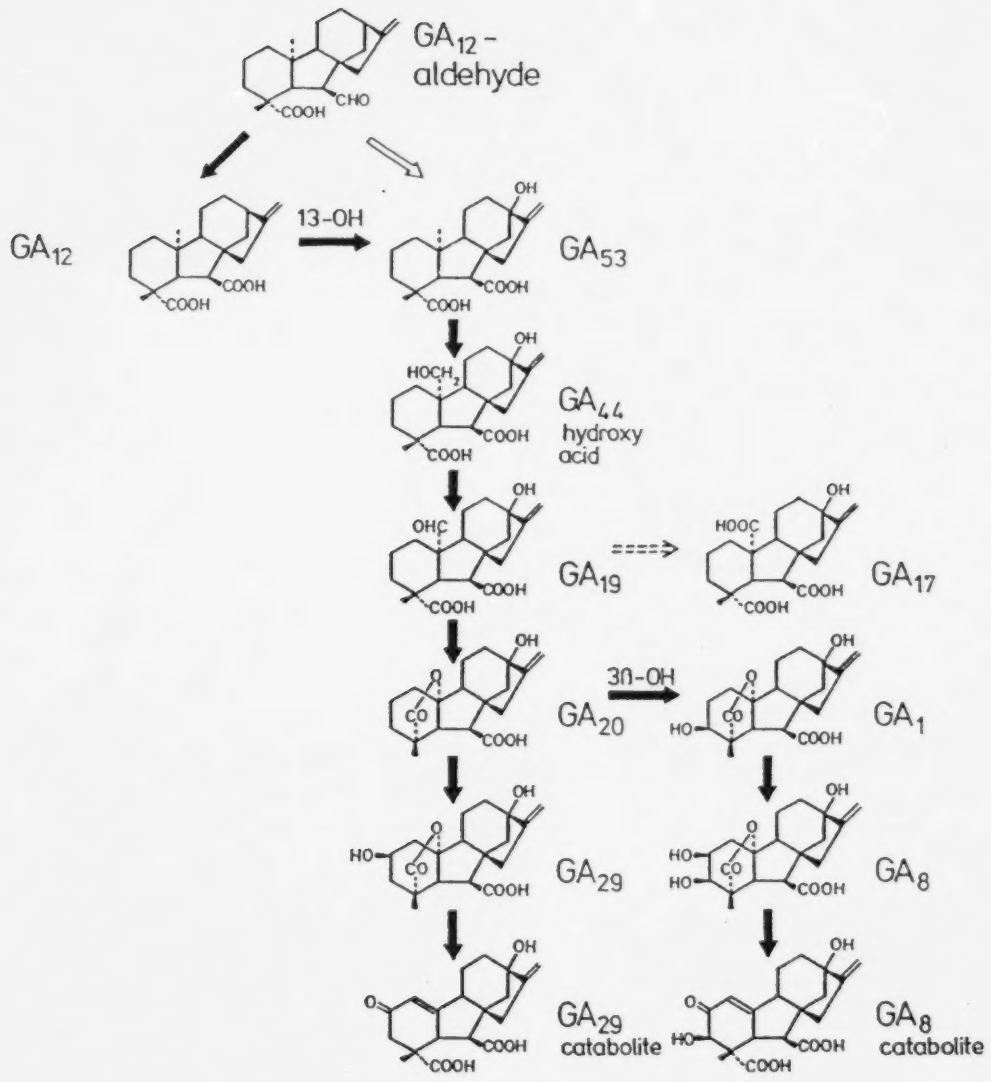

Figure 1.1. The early 13-hydroxylation GA biosynthetic pathway in *Pisum sativum* L. (Graebe, 1987).

Oxidation (GA_{53} -> GA_{44} -> GA_{19}) and elimination of C-20 (GA_{19} -> GA_{20}) are thought to be other sites of regulation. In spinach, a long day (LD) rosette plant, bolting in response to long days is associated with increased rates of GA_{53} and GA_{19} oxidase activities (Gilmour et al., 1986). Furthermore there is evidence suggesting that the GA_{53} and GA_{19} oxidase activities are down-regulated in maize (*Zea mays*) seedlings as a result of GA action in a type of feedback control (Hedden and Croker, 1992).

cDNA clones encoding GA 20-oxidases from pumpkin (Lange et al., 1994), *Arabidopsis* (Phillips et al., 1995; Xu et al., 1995), spinach (Wu et al., 1996) and pea (Garcia-Martinez et al., 1995; Proebsting et al., 1996; Lester et al., 1996) have been isolated. Heterologous expression of the GA 20-oxidase cDNA clones from pumpkin, *Arabidopsis*, and spinach in *Eschericha coli* has shown that their fusion proteins can catalyze the biosynthetic sequences GA_{53} -> GA_{44} -> GA_{19} -> GA_{20}, and GA_{19} -> GA_{17}, suggesting that GA 20-oxidase is a multifunctional enzyme responsible for the oxidation and elimination of C-20. The fusion protein of pumpkin GA 20-oxidase converted mostly GA_{19} to GA_{17}, while in *Arabidopsis* and spinach, GA_{19} was converted mainly to GA_{20}. These findings correlate with the observation that *Arabidopsis* and spinach produce predominantly C-19 GAs (Talon et al., 1990; Talon et al., 1991), whereas developing pumpkin seeds produce biologically inactive C-20 tricarboxylic acids as major products (Lange et al., 1994).

In spinach and *Arabidopsis* expression of GA 20-oxidase has been shown to be regulated by photoperiod (Xu et al., 1995; Wu et al., 1996). GA 20-oxidase transcript levels in spinach were enhanced after plants were transferred from short to long days, with the highest level of expression in the shoot tips and elongating stems (Xu et al., 1995).

Previous studies with spinach by Zeevaart's lab established that the activities of GA_{53}- and GA_{19}-oxidase, catalyzing the first oxidation, and the loss of C-20, are light

dependent, whereas GA_{44}-oxidase, which catalyzes the second oxidation at C-20, is not (Gilmour et al., 1986). Furthermore, Gilmour et al. (1987) could partially separate GA_{53}-oxidase and GA_{19}-oxidase activities from GA_{44}-oxidase activity by anion-exchange HPLC. Wu et al. (1996) argues that these findings appear to be in conflict with the finding that all three reactions (GA_{53} -> GA_{44} -> GA_{19} -> GA_{20}) are catalyzed by one enzyme with a single active site. Zeevaart's group proposes the possibility that there may be two enzymes that convert GA_{44} to GA_{19}: the multifunctional GA 20-oxidase that catalyzes the sequential oxidation of C-20 (GA_{53} -> GA44 -> GA_{19} -> GA_{20}) and a second enzyme catalyzing the single step, GA_{44} to GA_{19} (Wu et al., 1996).

Phillips et al. (1995) have isolated three GA 20-oxidase cDNA clones from *Arabidopsis*. The three GA 20-oxidase cDNAs hybridized to mRNA species with tissue-specific patterns of accumulation: one GA 20-oxidase was expressed in stems and inflorescences, another one in inflorescences and developing siliques, and the third one in siliques only. Garcia-Martinez et al. (1995) and Proebsting et al. (1996) isolated GA 20-oxidase cDNA clones from pea. Expression of the pea GA 20-oxidase gene reported by Proebsting et al. (1996), appears to be more abundant in leaves and stipules than in internodes, petioles and apical buds. Transcript levels increased as the foliage, internodes and petioles expanded and peaked at full expansion of these organs. GA 20-oxidase transcripts were also present in seeds and pods, but only during the first week of fruit development (Proebsting et al, 1996).

Given that GA_1 is probably the active GA in most plants, it is possible that the formation of GA_1 by the 3ß-hydroxylation of GA_{20} is regulated by the abundance of GA 3ß-hydroxylase transcripts. Chiang et al. (1995) have cloned an *Arabidopsis* gene named *GA4* that encodes a putative GA 3ß-hydroxylase. The transcripts for the *GA4*-encoded3ß-

hydroxylase accumulate to relatively high levels in siliques and to lower levels in flowers, roots, and leaves, but the significance of these results is not yet clear.

Several findings support the view that bioactive GAs may control their own synthesis through a negative feedback mechanism on the mRNA levels of GA biosynthetic genes. Treating *Arabidopsis* leaves with GA_4 (a down-stream product of GA 20-oxidase; Xu et al., 1995) or GA_3 (Phillips et al., 1995) caused GA 20-oxidase mRNA levels to decrease. Overexpression of the *GA4*-encoded 3ß-hydroxylase in *Arabidopsis* leaves of the ga4-1 mutant, was repressed when exogenous GA_3 was applied (Chiang et al., 1995).

In summary, the early 13-hydroxylation GA biosynthetic pathway is active in pea: GA_{12} -> GA_{53} -> GA_{44} -> GA_{19} -> GA_{20} -> GA_1. GA 20-oxidase, a multifunctional enzyme, catalyzes the biosynthetic sequences GA_{53} -> GA_{44} -> GA_{19} -> GA_{20} and GA_{19} -> GA_{17}. Increasing evidence suggests that regulation of GA 20-oxidase gene expression plays an important role in the GA biosynthesis.

Role of Auxins and Gibberellins in Pea Fruit Development

Gibberellins

GA_1, GA_3, GA_8, GA_{19}, GA_{20} and GA_{29} have been identified in ovules and pods from 4-day-old pollinated ovaries of pea; and GA_{17}, GA_{81} and GA_{29}-catabolites were identified additionally in pods (Garcia-Martinez et al., 1987; Sponsel and Reid, 1992; Gaskin et al., 1985; Garcia-Martinez et al., 1991). High concentrations of GA_1 and GA_3 in pollinated ovaries have been found during rapid pod elongation (Garcia-Martinez et al., 1991). The source of GAs involved in fruit development is unclear. The GAs which promote fruit growth in pea could be synthesized in either the pericarp tissue, the developing ovules, or imported from the maternal plant. Garcia-Martinez's group (1991)

has suggested that fertilized ovules regulate the fruit-set and pericarp development in pea by supplying GAs to pericarp tissue. However, in the pea *na* mutant, the production of biologically active gibberellins is blocked in shoots and pericarp tissue, but not in developing seeds. The *na* pods contained little or no significant gibberellin-like activity despite the presence of relatively high levels of GAs in developing seed at the same time. These results suggest that there is little or no significant transport of biologically active gibberellins from developing seeds to the pericarp (Potts, 1986). In addition, maturing seeds have high amounts of GAs at 21 DAA (GA_{20} 4.8 µg/embryo; Sponsel, 1983), however pericarp growth has ceased by approximately 8 DAA.

An alternative hypothesis has been proposed that seeds may promote pericarp growth by maintaining GA biosynthesis in the pericarp tissue (Sponsel, 1982). Maki and Brenner (1991) examined the capacity of the pericarp tissue to produce gibberellins. Pea pericarp tissue has the capacity to metabolize [^{14}C]GA_{12} and [^{2}H]GA_{53} to [^{14}C]GA_{20} and [^{2}H]GA_{1}, respectively, but only when the seeds are present and the fruit is attached to the plant (Maki and Brenner, 1991, Ozga et al., 1992). By applying the substrate [^{14}C]GA_{12} to deseeded pericarp, Ozga et al. (1992) found that seed removal inhibited conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20}, while causing accumulation of radiolabeled and endogenous GA_{19}. These data suggest that the pericarp is unable to convert GA_{19} to GA_{20} in the absence of the seeds. 4-Cl-IAA (a naturally occurring auxin) can substitute for the seeds in pericarp growth (Reinecke et al., 1995) and, as suggested from data obtained by HPLC/TLC, in stimulation of converison of GA_{19} to GA_{20} (Ozga and Brenner, 1992).

Auxins

Auxins are a class of plant growth regulators defined by their biological activity. Pea fruit contain the auxins indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic acid (4-

Cl-IAA; Ozga et al., 1993). 4-Cl-IAA and its methyl ester were isolated from immature pea seeds in the late 1960's (Marumo et al., 1968a; Marumo et al., 1968b). 4-Cl-IAA is characterized by exceptionally strong hormonal activities compared to IAA; 10-fold more active for elongation of the *Avena* coleoptile, and 100-fold more active for hypocotyl swelling of the mung bean, as defined by the concentration required to reach maximum biological response (Marumo et al., 1974, Bottger et al., 1978). 4-Cl-IAA also induces very high ethylene evolution in pea cuttings, which lasts much longer than that produced by IAA treatment (Ahmad et al., 1987). These high biological activities of applied 4-Cl-IAA relative to IAA have been ascribed partly to 4-Cl-IAA's stability (resistance to peroxidase oxidation) *in vitro* (Marumo et al., 1974). 4-Cl-IAA and its methyl ester have been shown to occur also in immature seeds of *Vicia* and *Lathyrus* species (Engvild et al., 1978, 1980, Katayama et al., 1987, 1988; Hofinger and Bottger, 1979).

Pea seeds at 6 DAA contain high amounts of IAA (1,713 ng/g fwt) and 4-Cl-IAA (132 ng/g fwt). Seeds (6 DAA) contain 46 times higher concentrations of free 4-Cl-IAA than pericarp tissue (2.9 ng/g fwt; Ozga et al., 1993). The presence of 4-Cl-IAA in pericarp tissue and the presence of high levels of 4-Cl-IAA in seed tissue suggest that the seeds may be the source tissue for 4-Cl-IAA present in the pericarp. Reinecke et al. (1995) studied the effect of IAA, 4-Cl-IAA, and halogen-substitution of the indole ring on growth of pea pericarp. Applications of 4-Cl-IAA promote pericarp growth, the effect increasing with concentration from 1 to 100 μM, however IAA was ineffective in stimulating growth when tested from 0.1 to 100 μM. The effect of the position of the halogen on pericarp growth was examined by assaying the activities of 4-, 5-, 6-, and 7-chloro- and -fluoro-substituted IAA. In this pea pericarp assay, 4-Cl-IAA, and to a lesser extent, 5-Cl-IAA promoted growth, while all other derivatives were inactive or inhibitory. In pea stems and *Triticum* coleoptiles assays, IAA, 4-, 5-, 6- and 7-Cl-IAA and 5 F-IAA

were all active, although maximum activity was observed at different concentrations (Katekar and Geissler, 1983; Hoffmann et al., 1952; Sell et al., 1952).

Although the presence of 4-Cl-IAA in pea seeds has been known since the late 1960's, no specific function for this endogenous halogenated auxin has been found. In pea fruit, endogenous 4-Cl-IAA may have a specific role as a seed signal involved in the coordination of growth and development of the seed and surrounding pericarp tissue. One possible seed-regulatory mechanism in young pea fruit would be the export of 4-Cl-IAA from seeds to the pericarp where it stimulates GA biosynthesis; specifically the conversion of GA_{19} to GA_{20}, an apparent rate limiting step in the GA biosynthesis pathway. Previous experiments using the GA biosynthesis inhibitor paclobutrazol (blocks GA biosynthesis prior to GA_{12} aldehyde) suggest that 4-Cl-IAA may also have auxin effects on growth independent of its effect on GA biosynthesis (Brenner and Ozga, 1991).

GA and Auxin Perception

GAs and auxins mediate a large range of developmental and growth responses (Bardense et al., 1991; Evans et al., 1990; Fincher, 1989; Davies, 1995). Progress has been made towards the understanding of GA and auxin perception (Hooley et al., 1993; Venis and Napier, 1995) and their regulation of gene expression (Hagen, 1995; Rogers and Rogers, 1992; Rushton et al., 1992; Theologis, 1986). In spite of this progress, the sites of action and mechanism by which these two hormones elicit a physiological response in plants are still largely unknown. The proteins that perceive GAs and auxins, have been sought by many laboratories and the search has identified a few candidates. There is a substantial body of data supporting a model of auxin action in which auxin binds to an auxin binding protein (ABP1) on the cell surface inducing a signal cascade (Napier and Venis, 1995). Several groups have tried to purify proteins that bind gibberellins (gibberellin-binding proteins or GBPs) from various plant materials including pea epicotyls

(Lashbrook et al., 1987), cucumber hypocotyls (Yalpani et al., 1989) and the aleurone layers of oat seeds (Hooley et al., 1993). In spite of extensive studies, purification of GBPs so far has not been very successful.

Interactions between GAs and Auxins

Numerous reports exist of auxins and GAs synergistically affecting shoot and fruit development, however no definite data have been provided to explain the nature of their interaction. In several tissues auxin biosynthesis can be affected by GAs. Treatment with GAs frequently increases IAA levels in growing stems of pea (Law and Hamilton, 1984) and bean (Jindal and Hemberg, 1976), and there is considerable evidence that GA_3 can induce increased IAA biosynthesis in bean plants (Jindal and Hemberg, 1976), cabbage (Skytt-Andersen and Muir, 1969) and *Avena* coleoptiles (Valdovinos and Sastry, 1968). Furthermore, the GA biosynthesis inhibitor uniconazol decreased both IAA levels and stem growth in a wild-type pea and subsequent addition of GA_3 to uniconazol treated plants increased both growth and IAA (Law and Hamilton, 1989). More specifically, Kutacek (1985) has presented evidence for GA-enhanced indoleacetaldehyde oxidase activity and Law (1987) has argued in favor of a regulatory role of GA in generation of D-tryptophan as an IAA precursor. Others have found evidence for GA_3-enhanced D-tryptophan aminotransferase activity (McQueen-Mason and Hamilton, 1989).

Eeuwens and Schwabe (1975) were the first researchers to study the interaction of GAs and auxins in pea fruit development. They found that application of GA plus NAA (a synthetic auxin) had a synergistic effect on fruit growth. Similar results were observed when pericarp was treated with GA plus 4-Cl-IAA (Ozga and Reinecke, 1994).

From studies using the pea split-pericarp system (Ozga et al., 1992; Reinecke et al., 1995; Ozga and Brenner, 1992) a working hypothesis has been developed that envisions 2

regulatory roles for auxin in controlling fruit growth: (1) the export of 4-Cl-IAA from the seeds to the pericarp where it stimulates GA biosynthesis, and (2) a direct auxin effect of 4-Cl-IAA on pericarp growth (Reinecke et al., 1995; Ozga and Brenner, 1992).

Objectives

The goal of my thesis was to characterize further the biochemical and molecular basis of seed-induced pericarp growth in pea fruit. The specific objectives were to:

1. Confirm that seeds and/or 4-Cl-IAA regulate the conversion of GA_{19} to GA_{20} in pea pericarp by monitoring the metabolism of ^{14}C-GA_{19} in this tissue.
2. Understand how seeds and/or 4-Cl-IAA regulate the conversion of GA_{19} to GA_{20} in pea pericarp by examining the regulation of GA 20-oxidase gene expression. The product of this gene (GA 20-oxidase) can catalyze the multiple steps of oxidation and elimination of C-20 (GA_{53} -> GA_{44} -> GA_{19} -> GA_{20})
3. Assess at the protein level, the relative contribution of GA and 4-Cl-IAA to pea fruit development. *In vivo* protein labeling with [^{35}S]Met coupled with two dimensional PAGE was utilized to obtain a profile of hormonal and seed-induced polypeptide changes during early fruit development.
4. Use molecular markers to characterize mitotic activity and cell enlargement during early pea fruit development, and during GA and/or 4-Cl-IAA stimulated pea pericarp growth.

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Chapter 2

Seed and 4-Chloroindole-3-Acetic Acid Regulation of Gibberellin Metabolism in Pea Pericarp

INTRODUCTION

In pea (*Pisum sativum* L.), normal pod (pericarp) growth requires the presence of seeds (Eeuwens and Schwabe, 1975). Developing seeds (4 and 6 DAA) contain biologically active GA_1 and GA_3 (Garcia-Martinez et al., 1991), and the requirement for seeds for pericarp growth can be replaced by application of GAs (Eeuwens and Schwabe, 1975; Sponsel, 1982). Based on these observations, it has been assumed that the GAs biosynthesized by seeds are transported to the pericarp and regulate pericarp growth. However, an alternative hypothesis that seeds may promote pericarp growth by maintaining GA biosynthesis in the pericarp was proposed by Sponsel (1982). The early 13-hydroxylation pathway of GA biosynthesis is known to occur in pea seeds (Graebe, 1987): GA_{12} -> GA_{53} -> GA_{44} -> GA_{19} -> GA_{20} -> GA_1. Pea pericarp tissue has the capacity to metabolize [^{14}C]GA_{12} to [^{14}C]GA_{20} and [^{2}H]GA_{53} to [^{2}H]GA_1, but only when the seeds are present and the fruit is attached to the plant (Maki and Brenner, 1991; Ozga et al., 1992). Removal of seeds from the pericarp inhibited pericarp growth and the conversion of [^{14}C]GA_{12} to [^{14}C]GA_{20} while causing accumulation of radiolabeled and endogenous GA_{19} (Ozga et al., 1992). Deseeded pericarp contained no detectable GA_{20}, GA_1, or GA_8, whereas pericarp with seeds contained endogenous and radiolabeled GA_{20} and endogenous GA_1. These data suggest that seeds regulate a key step in the GA biosynthesis pathway, i.e. conversion of GA_{19} to GA_{20}. Developing pea fruit (6 DAA) also contain the auxins IAA and 4-Cl-IAA (Ozga et al., 1993), and exogenous 4-Cl-IAA promotes growth in deseeded pea pericarp (Reinecke et al., 1995). Preliminary experiments in which

[^{14}C]GA_{12} was used suggested that 4-Cl-IAA can substitute for the seeds in the conversion of putative GA_{19} to GA_{20} in pea pericarp (Ozga and Brenner, 1992; metabolism monitored by a TLC-radiochemical plate scanner). In the present study, [^{14}C]GA_{19} metabolism was monitored in pea pericarp to investigate further seed and auxin regulation of GA biosynthesis in pericarp, specifically conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20}. This study confirms results of previous studies using [^{14}C]GA_{12} that seeds regulate the conversion of GA_{19} to GA_{20} and verifies preliminary data that 4-Cl-IAA can stimulate the conversion of GA_{19} to GA_{20} in deseeded pea fruit.

MATERIAL AND METHODS

Plant Material, Chemicals, and Treatments

Seeds of *Pisum sativum* L., line I_3 (Alaska type) were germinated in 20-cm pots (three plants per pot) containing a 1:1 ratio of Metro-Mix:sand (W.R. Grace and Co., Toronto, Canada). The plants were grown in a growth chamber (Conviron, Ashville, NC) at 19/17^{o}C (day/night) with cool-white fluorescent and incandescent light and a 16-h photoperiod. The lights were kept 30 cm above the top of the canopy with an average photon flux density of 450 $\mu Em^{-2}s^{-1}$. One fruit per plant (at the third to fifth flowering node) was used for treatments, and subsequent flowers were removed as they developed. Terminal apical meristems of plants were intact, and the pericarp remained attached to the plant during the entire experiment. To remove the seeds, a split-pod technique was used as described by Ozga et al. (1992). Briefly, pericarps of 2 DAA (15-20 mm) ovaries (pericarp plus seeds) were left intact (intact treatment) or split down the dorsal suture, either without disturbing the seeds (SP treatment) or with removal of the seeds (SPNS treatment). Splitting of the pericarp and seed removal were completed 24 h prior to radiolabel application. 4-Cl-IAA (50 μM in 0.1% Tween 80 solution, 30 μL) was applied twice to the

inside surface of the pericarp (endocarp) immediately after seed removal and approximately 20 min prior to [^{14}C]GA_{19} application.

[^{14}C]GA_{19} was applied in 2 µL of 50% aqueous ethanol to the inside surface of both sides of the pericarp. Treated pericarp tissues (seeds were removed if present) were harvested onto dry ice 2, 4, 8, and 24 h after [^{14}C]GA_{19} application and stored at -80°C until extraction. In a preliminary experiment 20,000 dpm of [^{14}C]GA_{19} per pod were used to show that metabolic profiles and ratios of free labeled-GA products were similar to those of pods receiving 80,000 dpm; therefore, to obtain more labeled metabolite, 80,000 dpm per pod were used for all experiments in this study.

The following work was done by Drs Twitchin and Mander, Australian National University: 17-[^{14}C]GA_{19} (specific activity of 54mCi/mmol) was prepared from GA_3 by means of a 15-step sequence based on established methodology (Dawe et al., 1985a, 1985b). Following oxidative cleavage of the 17-methylene group (cf. Lombardo et al., 1981), the radiolabel was introduced by means of the Wittig procedure using [^{14}C]methyltri-phenylphosphonium iodide with a specific activity of approximately 55mCi/mmol (obtained from Amersham) on the 17-nor-16-ketone (cf. Lombardo et al., 1980). Full details of the synthesis will be published elsewhere.

Extraction Procedure and Partitioning

Radiolabeled pericarps (two per sample) were homogenized in cold 80% methanol (10 mL per sample) containing 10 mg/L butylated hydroxytoluene using a Polytron homogenizer. [^{3}H]GA_4 (9000 dpm) was added to the tissue at the time of homogenization as an external standard for determination of recovery. After approximately 1 h, the extracts were centrifuged for 30 min at 10,000*g*. The supernatant was removed, and the residue was immediately resuspended in 10 mL of the solvent used for homogenization and gently shaken 12 to 16 h in darkness at 4°C. The extracts were centrifuged for 30 min at

10,000*g*, and the combined supernatants were reduced to the aqueous phase using a vacuum concentrator (Savant, Farmingdale, NY). After the pH was adjusted to 8.0 with NH_4OH (0.1 N) the aqueous extracts were partitioned against n-hexane (5 mL) four times. The aqueous fraction was then adjusted to pH 3.0 with HCl (0.1 N) and partitioned against the ethyl acetate (5 mL) five times. The combined ethyl acetate extracts were partitioned against 5% (w/v) aqueous $NaHCO_3$ (5 mL) four times. The combined $NaHCO_3$ extracts were adjusted to pH 3.0 and partitioned against ethyl acetate (5 mL) five times. The ethyl acetate extracts were combined and evaporated to dryness under vacuum.

Chromatography

SiO_2 partition chromatography was performed on each extract to further separate free GAs from GA conjugates (adapted from Rood et al., 1983). Briefly, the samples were dissolved in 0.5 mL of methanol:ethyl acetate:H_2O (50:50:1, v/v) and placed at the top of a SiO_2 column (1 g; 20% H_2O by weight) which had been slurried and packed with formic acid-saturated hexane:ethyl acetate (95:5, v/v). Free GAs were eluted from the column with 15 mL of formic acid-saturated hexane:ethyl acetate (5:95, v/v) and brought to dryness under vacuum.

The free GA fractions were resuspended in 400 µL of 20% methanol, passed through 0.45-µm nylon filters prior to injection onto a 4.5 x 250 mm Spherisorb C_{18} column (5 µm; Phase Sep, Norwalk, CT). The samples were eluted at a flow rate of 1 mL/min using a linear gradient of 0.01% TFA (solvent A) and methanol (solvent B) as follows: 20% solvent B for 1 min, gradient to 100% solvent B in 45 min, and isocratic 100% solvent B for 5 min. Radioactivity in the effluent was monitored using a flow-through radiochemical detector (Beckman 171). Radioactive fractions eluting at the retention times of GA_{29} (11.9 min), GA_1 (16.9 min), GA_5 (23.2 min), and GA_{20} (24.4 min) were collected and dried. These putative ^{14}C-GAs were methylated using

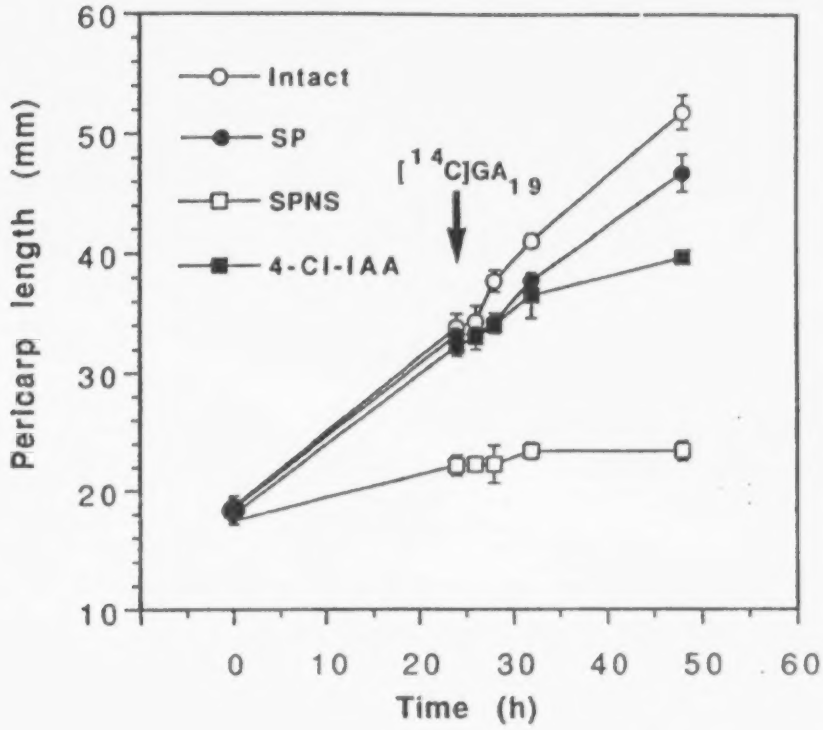
diazomethane and rechromatographed as their methyl esters by C_{18} HPLC, using the same solvent system.

The [^{14}C]GA_{20} and [^{14}C]GA_{29} methyl esters were converted to trimethylsilyl ether derivatives (Gaskin and MacMillan, 1991). GA identity was confirmed by Dr. Ozga, University of Alberta, using GC-MS-SIM. Mass spectral analyses of derivatized samples were performed using a Hewlett-Packard model 5972A Mass Selective Detector equipped with an HP-5 MS column (30 m x 0.25 mm x 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1 $mLmin^{-1}$. The samples were injected on-column with the initial column temperature at 50°C for 2 min, followed by temperatures programming at 10°C min^{-1} to 150°C and then 3°C min^{-1} to 300°C. SIM of three prominent ions for each GA of interest and KRI data were used for confirmation of ^{14}C-GA identity.

RESULTS

The lengths of pericarps with seeds (intact and SP treatment) and of deseeded pericarps treated with 4-Cl-IAA increased during the 24-h period of incubation with [^{14}C]GA_{19} (Fig. 2.1). The linear effect accounted for 95% of the variation during the [^{14}C]GA_{19} incubation time and was highly significant (F test; $P < 0.0001$). The rate of pericarp growth with seeds (intact and SP treatment) was significantly higher than that of deseeded pericarp treated with 4-Cl-IAA (linear interaction significant at $P < 0.01$). When seeds were removed (SPNS control), growth of pericarp was significantly less ($P < 0.01$) than growth of pericarp with seeds and deseeded pericarp treated with 4-Cl-IAA. SPNS pericarps were still viable, however, since application of hormones (GA_3 plus 4-Cl-IAA, 50 µM) to pericarp 24 and 48 h after seed removal stimulated growth to 79% (27.0 ± 2.9 mm) and 48% (8.3 ± 3.5 mm), respectively, of that of pericarp treated immediately after seed removal (growth = increase in length after 24 h [34.3 ± 1.4 mm] or 48 h [17.3 ± 0.6 mm] until harvest at 9 DAA).

Figure 2.1. Effects of pericarp splitting (SP), of seed removal (SPNS), and of seed removal plus treatment with 4-Cl-IAA on pea pericarp growth. Arrow indicates time of $[^{14}C]GA_{19}$ application. Data are means ± SE (n = 6)


Intact
SP
SPNS
4-Cl-IAA
[14C]GA19
Pericarp length (mm)
Time (h)
10
20
30
40
50
60
0
10
20
30
40
50
60

Forty-nine to 76% of the [^{14}C]GA_{19} substrate remained unmetabolized after the 2-, 4-, and 8-h incubation periods in all treatments. After 24 h of incubation, 10 to 26% remained unmetabolized in the intact, SPNS, and SPNS plus 4-Cl-IAA treatments and 42% in the SP treatment.

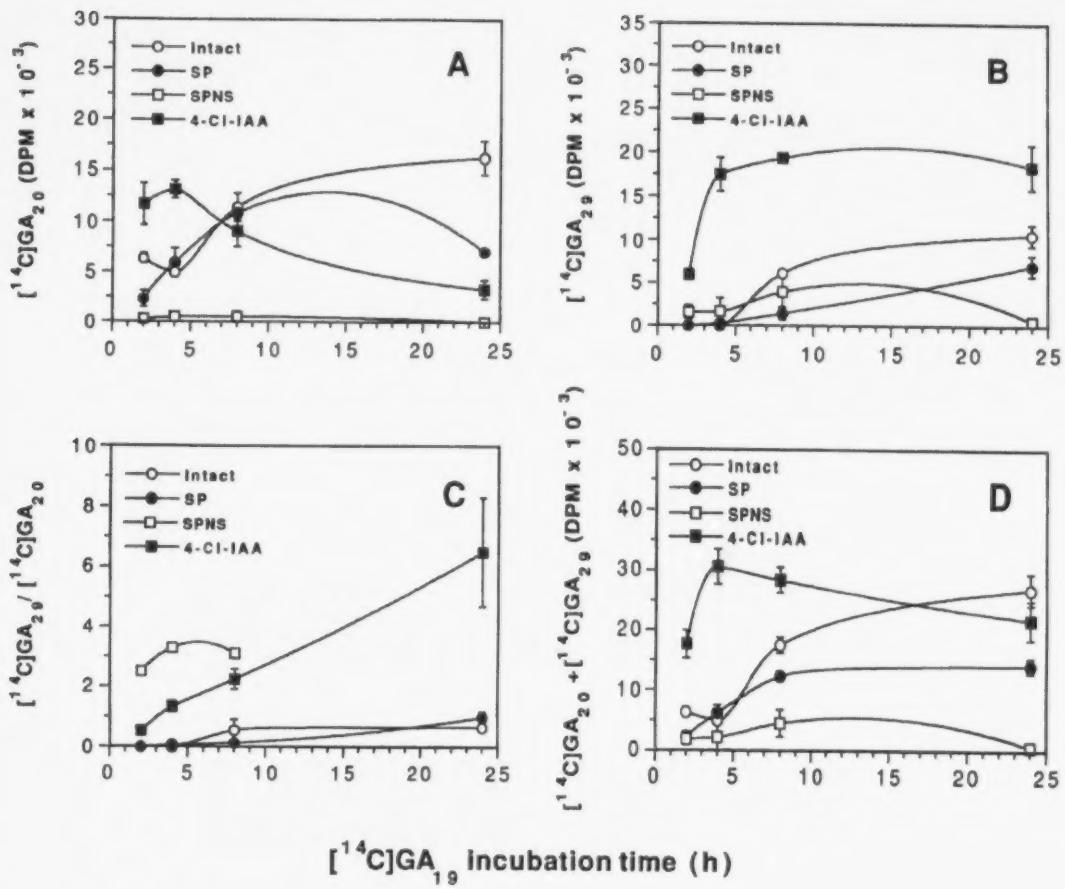
Pericarp with seeds (intact and SP treatment) exhibited similar patterns of [^{14}C]GA_{20} accumulation in the first 8 h of incubation with [^{14}C]GA_{19} (Fig 2.2A). After 24 h, [^{14}C]GA_{20} levels were significantly higher in the intact than in the SP treatment. Seed removal (SPNS) resulted in little or no accumulation of [^{14}C]GA_{20} (Fig. 2.2A). However, when deseeded pericarps were treated with 4-Cl-IAA, [^{14}C]GA_{20} levels were higher or similar to levels observed in pericarp with seeds (intact and SP treatment) after 2, 4, and 8 h of [^{14}C]GA_{19} incubation but significantly lower than in intact and SP after 24 h (Fig. 2.2A)

Little to no [^{14}C]GA_{29} was detected after 2 or 4 h of incubation with [^{14}C]GA_{19} in pericarp with seeds (intact and SP), but levels increased with time (Fig. 2.2B). In SPNS, [^{14}C]GA_{29} was detected at all incubation periods, but levels decreased to a minimum after 24 h of incubation. At all incubation periods, levels of [^{14}C]GA_{29} were significantly higher in deseeded pericarp treated with 4-Cl-IAA than in all other treatments.

The [^{14}C]GA_{20} and [^{14}C]GA_{29} fractions were pooled, methylated and rechromatographed on C_{18} HPLC. They exhibited single peaks at the same retention time as methylated [^{3}H]GA_{20} and [^{13}C-^{3}H]GA_{29} respectively. The identity of [^{14}C]GA_{20} and [^{14}C]GA_{29} was confirmed by GC-MS-SIM. The relative intensities of ions formed in the MS fragmentation of standard and sample GA-methyl-trimethylsilyl derivatives and KRIs are as follows: [^{14}C]GA_{20} (420 M^+, 100; 405, 29; 377, 88; KRI 2543); ^{2}H-GA_{20} standard (420 M^+, 100; 405, 18; 377, 84; KRI, 2543); [^{14}C]GA_{29} (508 M^+, 100; 493, 13; 391, 18; KRI, 2714); ^{2}H-GA_{29} standard (508 M^+, 100; 493, 13; 391, 18; KRI 2714).

Putative [^{14}C]GA_5 was detected in several samples of pericarp with seeds (intact and SP) and 4-Cl-IAA-treated deseeded pericarp (123-3244 dpm per sample at the first

Figure 2.2. Effects of pericarp splitting (SP), of seed removal (SPNS), and of seed removal plus treatment with 4-Cl-IAA on the biosynthesis of [^{14}C]GA_{20} (A), [^{14}C]GA_{29} (B), ratio of [^{14}C]GA_{29} to [^{14}C]GA_{20} (C), and [^{14}C]GA_{20} plus [^{14}C]GA_{29} (D) in pea pericarp incubated with [^{14}C]GA_{19}. Data are means ± SE (n = 3 samples; two pericarps per sample). In C, values for SPNS treatment at 2-, 4-, and 8-h incubation periods are based on one sample; the two remaining samples contained either [^{14}C]GA_{29} but no [^{14}C]GA_{20} or no [^{14}C]GA_{29} or [^{14}C]GA_{20}; after 24 h of incubation, [^{14}C]GA_{29} was detected in only one sample of three in the SPNS pericarp and [^{14}C]GA_{20} was not detected in any of these samples


A
B
C
D
Intact
SP
SPNS
4-Cl-IAA
[14C]GA20 (DPM x 10-3)
[14C]GA29 (DPM x 10-3)
[14C]GA29/[14C]GA20
[14C]GA20+[14C]GA29 (DPM x 10-3)
[14C]GA19 incubation time (h)

HPLC step). After the pooled putative [^{14}C]GA_5 fractions were methylated and rechromatographed on C_{18} HPLC, two ^{14}C-GA peaks of approximately the same size were detected. One peak occurred at the same retention time (27.4 min) as the methylated [^{3}H]GA_5 standard. The second peak eluted after the methylated [^{3}H]GA_5 peak (29.9 min), and its identity is unknown. Quantities of putative [^{14}C]GA_5 were not sufficient to be confirmed by GC-MS-SIM analysis.

Putative [^{14}C]GA_1 was not detected in any treatment throughout the 24-h incubation period with one exception: a small peak (827 dpm) at the retention time of GA_1 (16.9 min) was observed in one intact pericarp sample (24 h of incubation). Putative radiolabeled GA_3 and GA_8 were not detected in any treatment.

The 2ß-hydroxylation of GA_{20} to GA_{29} is widely recognized as a deactivation step, resulting in loss of GA activity (Hoad et al., 1982). The ratio of [^{14}C]GA_{29} to [^{14}C]GA_{20} is an indication of the relative amount of GA that has flowed out of the active GA pool at this step in the biosynthesis pathway. In pericarp with seeds (intact and SP treatment) the ratio remained less than 1 for the entire incubation period (Fig.2.2C). When seeds were removed (SPNS), the ratio was higher than 2 in the first 8 h of incubation and no [^{14}C]GA_{20} could be detected after 24 h. In contrast, the ratio of [^{14}C]GA_{29} to [^{14}C]GA_{20} in deseeded pericarp treated with 4-Cl-IAA increased linearly during the incubation period, reaching a ratio of 6.5 after 24 h of incubation.

Since [^{14}C]GA_{20} and [^{14}C]GA_{29} were the only major free ^{14}C-GA metabolites detected, the sum of these metabolites at any one incubation period is an estimate of the capacity of [^{14}C]GA_{19} to be metabolized through the GA_{19} to GA_{20} step. In general, a similar pattern of [^{14}C]GA_{19} metabolism of free ^{14}C-GA could be observed for both intact and SP treatments (Fig.2.2D). In both treatments [^{14}C]GA_{19} metabolism to [^{14}C]GA_{20} plus [^{14}C]GA_{29} increased over the initial 8-h of incubation. From 8 to 24 h of [^{14}C]GA_{19} incubation, the free ^{14}C-GA metabolites continued to increase in the intact treatment but remained constant in the SP treatment. The conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20} plus

[^{14}C]GA_{29} was low or not detectable in the SPNS control. Deseeded pericarp treated with 4-Cl-IAA showed the highest [^{14}C]GA_{19} conversion to free ^{14}C-GA metabolites in the first 8 h of incubation. After 24 h of incubation, [^{14}C]GA_{19} metabolism in 4-Cl-IAA treated deseeded pericarp was similar to that in the intact and SP treatments (Fig.2.2D).

DISCUSSION

We have shown that [^{14}C]GA_{19} is metabolized to [^{14}C]GA_{20} by pea pericarp when seeds are present. When the seeds are removed, the conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20} is greatly reduced or inhibited. These results confirm our previous results using [^{14}C]GA_{12}, a GA earlier in the pathway, that seeds are required for normal GA biosynthesis in the pericarp, specifically the conversion of GA_{19} to GA_{20} (Ozga et al., 1992). Pericarps with seeds (intact and SP treatment) exhibited a similar pattern of [^{14}C]GA_{20} accumulation during the first 8 h of [^{14}C]GA_{19} incubation, but levels of [^{14}C]GA_{20} in the SP treatment were significantly lower than in the intact pericarp after 24 h. In previous experiments (Ozga et al., 1992), inhibition of conversion of GA_{19} to GA_{20} did not appear to be an effect of wound ethylene produced as a result of seed removal, because treatment with silver thiosulfate, an ethylene action inhibitor, delayed ethylene-related physiological processes, such as tissue senescence and pericarp abscission, but did not overcome the inhibition of GA_{20} synthesis in deseeded pericarp. The lower levels of [^{14}C]GA_{19} in the SP treatment at the 24-h time period could be due to disruption of the vascular tissue when the pericarp was split down the dorsal suture.

We have also shown that 4-Cl-IAA can stimulate the conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20} in deseeded pea pericarp. The presence of 4-Cl-IAA in pea seeds has been known since the late 1960s (Marumo et al., 1968). However, no specific function for this endogenous halogenated auxin has been found. Reinecke et al. (1995) found that exogenous 4-Cl-IAA promoted growth in deseeded pea pericarp, with the effect increasing

with concentration from 1 to 100 μM, but IAA was ineffective in stimulating growth when tested from 0.1 to 100 μM. Reinecke et al. (1995) also examined the effect of the position of the halogen on pericarp growth by assaying the activities of 4-, 5-, 6-, and 7-chloro- and fluoro-substituted IAA. The position and type of the halogen dramatically affected auxin activity, with the natural product 4-Cl-IAA being most effective. In our system, endogenous 4-Cl-IAA might play a specific role as a seed signal by stimulating GA biosynthesis in the pericarp. 4-Cl-IAA stimulation of [^{14}C]GA_{19} metabolism to [^{14}C]GA_{20} plus [^{14}C]GA_{29} was significantly higher 2, 4, and 8 h after hormone 4-Cl-IAA application in deseeded pericarp than in pericarp with seeds, even though the rate of pericarp growth was significantly higher in pericarp with seeds than in deseeded pericarp treated with 4-Cl-IAA during this period. This experiment provides evidence that 4-Cl-IAA's effect on [^{14}C]GA_{19} metabolism is not simply an indirect effect of 4-Cl-IAA in maintaining pericarp growth. One possible seed regulatory mechanism in young pea fruit could be the export of 4-Cl-IAA by seeds to the pericarp, where it stimulates synthesis or activity of GA 20-oxidase, an apparently rate-limiting step in the GA biosynthesis pathway. 4-Cl-IAA also stimulates pericarp growth independently of its effect on GA metabolism, as shown by paclobutrazol (GA biosynthesis inhibitor) treatments (Brenner and Ozga, 1991). Increasing evidence suggests that the conversion of GA_{19} to GA_{20} is an important regulatory step in GA biosynthesis (Metzger and Zeevaart, 1980, 1982; Gilmour et al., 1986). Lange et al. (1994) have recently isolated the gene for GA20-oxidase from pumpkin endosperm. This enzyme catalyzes the oxidation of GA_{53} -> GA_{44} -> GA_{19} and the loss of the carbon-20 of GA_{19} to form GA_{20}. Further research is necessary to determine how GA 20-oxidase, an enzyme catalyzing multiple steps in a pathway, can be regulated specifically at its penultimate step.

Our data suggest that the 2ß-hydroxylation of GA_{20} to GA_{29} in the pericarp may also be seed regulated. The SPNS control and 4-Cl-IAA-treated deseeded pericarp exhibited a higher ratio of [^{14}C]GA_{29} to [^{14}C]GA_{20} than pericarp with seeds, suggesting

that conversion of GA_{20} to GA_{29} in the pericarp may be repressed by seeds or that the 4-Cl-IAA treatment results in a pulse of GA_{20} synthesis and a subsequent high production of GA_{29}. Although the ratio of [^{14}C]GA_{29} to [^{14}C]GA_{20} in 4-Cl-IAA-treated deseeded pericarp is higher than in pericarp with seeds after 2, 4, and 8 h of incubation, the amount of [^{14}C]GA_{20} was also greater in the 4-Cl-IAA-treated deseeded pericarp during this period. These data suggest that, although a greater amount of GA is flowing out of the active GA pool at this step in the 4-Cl-IAA treated tissue, a significant level of potentially active GA_{20} is still present.

Metabolism of [^{14}C]GA_{19} to putative [^{14}C]GA_1 was not observed in pea pericarp during the 24-h [^{14}C]GA_{19} incubation period. Maki and Brenner (1991) reported metabolism of [^{2}H]GA_{53} to [^{2}H]GA_1 after a 48-h incubation period in pea pericarp with seeds.

Putative [^{14}C]GA_5 was observed as a product of [^{14}C]GA_{19} metabolism in pericarp tissue with seeds and deseeded pericarp treated with 4-Cl-IAA. Fujioka et al. (1990) established that the biogenetic origin of GA_3 was GA_{20} via GA_5 in maize shoots (GA_{20} -> GA_5 -> GA_3). A similar pathway may exist in pea pericarp tissue . GA_3 is an endogenous GA in pea pericarp (Garcia-Martinez et al., 1991), but its biogenetic origin in this tissue is unknown. The detection of putative [^{14}C]GA_5 in pea pericarp suggest that the pericarp has the capacity to synthesize GA_5, the precursor to GA_3 in maize shoots.

We have shown that conversion of GA_{20} to GA_{29} may be seed regulated and that 4-Cl-IAA can substitute for the seeds in the conversion of GA_{19} to GA_{20}. These data support the hypothesis that the presence of seeds maintains GA production in the pericarp. Transmittable seed factors, such as 4-Cl-IAA, may be responsible for stimulating GA biosynthesis in the pericarp. The sink strength of the seeds may also be involved in maintaining pericarp GA biosynthesis. The pea split-pericarp growth system will allow us to further investigate how auxin 4-Cl-IAA and GA action and biosynthesis are linked and independent in pea fruit growth.

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Chapter 3

Seed and 4-Chloroindole-3-Acetic Acid Regulation of Gibberellin 20-Oxidase Expression in Pea Pericarp

INTRODUCTION

In pea (*Pisum sativum* L.), normal pericarp growth requires the presence of seeds (Eeuwens and Schwabe, 1975). Removal or destruction of the seeds 2-3 DAA results in slowing of pericarp growth and subsequent abscission (Eeuwens and Schwabe, 1975; Ozga et al., 1992). The effect of seeds on fruit development is assumed to involve plant hormones (Eeuwens and Schwabe, 1975; Gillaspy et al., 1993). Developing pea seeds contain GAs (biologically active GA_1 and GA_3; Garcia-Martinez et al., 1991) and auxins (4-Cl-IAA and IAA; Marumo et al., 1968; Ozga et al., 1993), and the requirement for seeds for pericarp growth can be replaced by application of GAs (Eeuwens and Schwabe, 1975) or 4-Cl-IAA (Reinecke et al., 1995).

Results obtained using a split-pericarp system suggest that seeds and 4-Cl-IAA can regulate a key step in the early 13-hydroxylation GA biosynthesis pathway in the pericarp, the conversion of GA_{19} to GA_{20} (Ozga et al., 1992; van Huizen et al., 1995).

Recently cDNA clones encoding GA 20-oxidases from pumpkin (Lange et al., 1994), *Arabidopis* (Phillips et al., 1995; Xu et al., 1995), spinach (Wu et al., 1996), and pea (Garcia-Martinez et al., 1995; Proebsting et al., 1996, Lester et al., 1996) have been isolated. Heterologous expression of the pumpkin, *Arabidopsis*, and spinach GA 20-oxidase cDNAs in *Escherichia coli* has shown that their fusion proteins catalyze the biosynthetic sequence GA_{53} -> GA_{44} -> GA_{19} -> GA_{20} and GA_{19} -> GA_{17}. These data suggest that a single protein is responsible for the sequential oxidation and elimination of C-20. In spinach and *Arabidopsis*, expression of GA 20-oxidase has been shown to be

regulated by photoperiod (Xu et al., 1995; Wu et al., 1996). GA 20-oxidase transcript levels were enhanced in leaves in *Arabidopsis*, and in stems, shoot tips and petioles in spinach, after plants were transferred from short to long days. In spinach the highest levels of expression occurred in the shoot tip and elongating stems. These results were consistent with the findings that the activities of the enzymes oxidizing GA_{53} and GA_{19} increased in these tissues in long days (Gilmour et al., 1986). GA 20-oxidase transcript levels in *Arabidopsis* decreased dramatically after GA application (Phillips et al., 1995; Xu et al., 1995), suggesting end-product repression in the GA biosynthesis pathway.

To further understand how seeds and 4-Cl-IAA regulate GA biosynthesis in pea pericarp at the molecular level, we have studied the expression of GA 20-oxidase in this tissue using northern blot analysis. Our data suggest that the the conversion of GA_{19} to GA_{20} in pea pericarp is controlled by seeds and 4-Cl-IAA, at least in part by regulating GA 20-oxidase mRNA levels in this tissue.

MATERIAL AND METHODS

Plant Material and Treatments

Plants of *Pisum sativum* L., line I_3 (Alaska-type) were grown in a 16-h photoperiod as previously described (van Huizen et al., 1995). One fruit per plant (at the third to fifth flowering node) was treated, and subsequent flowers were removed as they developed. Terminal apical meristems were intact and the pericarp remained attached to the plant during the entire experiment. To remove the seeds, a split-pericarp technique was used as described by Ozga et al. (1992). Briefly, pericarps of 2 DAA (15-22 mm) ovaries (pericarp + seeds) were split down the dorsal suture 1 h prior to the 8 h dark period, either without disturbing the seeds (SP treatment) or the seeds were removed immediately (SPNS treatment). Pericarps were treated with GA_3 and/or 4-Cl-IAA (30 µL, 50 µM in 0.1%

Tween 80) 12 h after deseeding, harvested 2, 4, 8, 12, and 24 h after hormone treatment and placed immediately into liquid N_2 and stored at -70°C until RNA extraction. The SP and SPNS controls were treated with 0.1% Tween 80 12 h after deseeding and harvested as described above. All treatments were applied to the inside surface (endocarp) of the pericarp. High humidity was maintained by enclosing the fruits in clear plastic bags throughout the duration of the experiment. Statistical analysis was performed by Roisin McGarry using The General Linear Model of SAS 6.10 (SAS Institute Inc, Cary, USA) following a completely randomized design.

RNA Isolation and PCR cloning

For each sample, 3 pods or approximately 30 seeds were ground in liquid N_2 and a 0.3 - 0.5 g subsample was used for RNA extraction. Total RNA was extracted following the Trizol (GibcoBRL) procedure based on Chomczynski and Sacchi (1987), with two additional chloroform extractions after the first chloroform extraction to remove polysaccharides.

To amplify a 690 bp fragment of the GA 20-oxidase from pea (Garcia-Martinez et al., 1995) an antisense degenerate primer (A) corresponding to the residues WHSIRPN and a sense degenerate primer (B) corresponding to the residues DEKPCMN were synthesized:

(A) 5'-TT[TA]GG[CT]CT[TAG]AT[TAG]GA[AG]TGCCA-3'

(B) 5'- GA[TC]GA[GA]AA[GA]CC [AT]TG[TC]ATGAA-3'

The first strand cDNA was reverse transcribed from 0.2 µg of total RNA from 6 DAA seeds or from 2 DAA pericarp with 200 units of SuperScript™RT (GIBCO-BRL) in a 20 µL volume containing 1X 1st strand buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl), 0.5 µM of primer B, 25 µM DTT and 25 µM dNTPs for 60 min at 37°C. Four µL of first-strand cDNA was amplified in a 50 µL volume containing 1X PCR buffer

(50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 1 μM of primer A and B, 2.5 units of *Taq* DNA polymerase (GIBCO-BRL), 250 μM dNTPs and 4 mM $MgCl_2$. The reaction mixture was heated to 94°C for 5 min, and then subjected to 40 cycles of 94°C for 1 min, 56°C for 2 min, and 72°C for 1 min. A final extension was performed at 72°C for 5 min. The products were separated by agarose gel electrophoresis and analyzed using ethidium bromide staining. The PCR reaction produced approximately 8 bands including the 690 bp product from both the pericarp and seed samples. The more abundant seed product was purified using a Geneclean II kit (BIO 101, Vista, USA), taken up in 100 μL H_2O, and 1 μL was used as a substrate in a second round of PCR under the conditions described above. The amplified product was again isolated from an agarose gel, polished with *Pfu* Polymerase (Stratagene) and ligated into pCR-Script^{TM}SK(+) (Stratagene). The 690 bp cDNA cloned in pCR-Script^{TM}SK(+) was transformed into *Escherichia coli* strain XL1 Blue.

DNA Sequence Analysis

Dye terminator sequencing of the PCR-generated fragments cloned into pCR-Script^{TM}SK(+) was performed using an Applied Biosystems 373A automated sequencing system. The DNA sequence analysis was performed using the ABA Prism version 2.11 program (Applied Biosystems).

Northern Blot Analysis

For northern blotting, total RNA (30 μg per sample) was denatured in 2.2 M formaldehyde/50% formamide and fractionated on a 1.2% agarose/2.2 M formaldehyde gel using a 20 mM MOPS buffer (pH 7.0; Maniatis et al., 1982) and transfered to Nylon membranes (Zeta-Probe GT, Bio-Rad) with 10X SSC. Equal loading and RNA integrity

were ascertained by ethidium bromide staining of ribosomal RNA bands prior to transfer. Membranes were baked for 2 h at 80°C under vacuum. RNA probes radiolabeled with [^{32}P]CTP were generated by *in vitro* transcription with T3 RNA polymerase according to the supplier's instructions (Riboprobe System, Promega) using the linearized plasmid as template, after digestion with *Sma* I. Prehybridization and hybridization of blots were performed at 50°C in a solution containing 60% formamide, 1X SSPE, 0.5% blotto, 10% dextran sulphate, 1% SDS, and 0.25 mg/mL denatured salmon sperm DNA. Blots were hybridized for 18 h with the labeled probe, then washed twice at room temperature for 15 min in 2X SSC, 0.1% SDS, and once at 70°C in 0.1X SSC, 1% SDS. Blots were stripped in 0.1X SSC and 0.5% SDS at 95°C for 20 min. As a loading control, all blots were also probed with an *Arabidopsis thaliana* actin probe (clone pATC-4 from Dr. R.J. Ferl, University of Florida, Gainesville). A [^{32}P]dATP random-primed actin cDNA probe was synthesized using the random primers DNA labeling system (GIBCO-BRL) according to the manufacturers instructions. Membranes were prehybridized, and then hybridized with the actin probe at 65°C in 0.5 M NaH_2PO_4 (pH 7.2), 7% SDS, and 1 mM EDTA for 18 h. Blots were washed twice for 30 min in 40 mM $NaHPO_4$ (pH 7.2), 5% SDS, 1 mM EDTA and twice for 30 min in 40 mM $NAHPO_4$ (pH 7.2), 1% SDS, 1 mM EDTA. For autoradiography, the membranes were exposed to Kodak X-Omat AR film at -70°C. The amount of labeled antisense RNA hybridization to the RNA blot was quantitated by scanning the autoradiogram with an imaging densitometer (Bio-Rad). For the 2 DAA and the 12 h after deseeding samples, one extraction of 12-15 pericarps for each time period, was performed and these samples were used as standards for all blots. The value at 2 DAA on each autoradiograph was designated 100% and all other signals were calculated relative to that sample.

RESULTS

GA_3- and 4-Cl-IAA-Stimulated Pericarp Growth

The length of pericarps with seeds (SP) and deseeded pericarps treated with 4-Cl-IAA, GA_3, and 4-Cl-IAA plus GA_3, increased during the 24 h period after hormone or 0.1% Tween 80 treatment (Fig. 3.1). The linear effect accounted for more than 95% of the variation during this period for these treatments and was highly significant (F test; $P < 0.0001$). When seeds were removed (SPNS), the rate of pericarp growth was significantly less (LSD; $P < 0.05$) than growth of pericarp with seeds (SP) and deseeded pericarp treated with 4-Cl-IAA, GA_3, and 4-Cl-IAA plus GA_3.

PCR Amplification of GA 20-oxidase Fragment using Degenerate Primers

The degenerate primers used for PCR to amplify the GA 20-oxidase sequence from pea seeds (6 DAA) were designed based on the amino acid sequence of the pea seed GA 20-oxidase (Garcia-Martinez et al., 1995). The primers yielded a product of expected size (690 bp). The PCR-amplified product was cloned into pCR-Script^{TM}SK(+) and sequenced. Sequence analysis was performed by Richard Chai, University of Alberta. The PCR product was 98.6% homologous to the pea GA 20-oxidase cDNA clone isolated by Garcia-Martinez et al. (1995).

Effect of Seeds and Hormones on GA 20-Oxidase Gene Expression

The effect of seeds and hormones (4-Cl-IAA and GA_3) on the expression of GA 20-oxidase in pea pericarp was investigated over a 36 h treatment period (Fig. 3.2 and 3.3). GA 20-oxidase mRNA levels in pericarp with seeds remained relatively stable in the

Figure 3.1. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with GA_3 (GA), 4-Cl-IAA (4-Cl),and GA_3 plus 4-Cl-IAA (GA+4-Cl) on pea pericarp growth. Two DAA pericarps were deseeded 12 h prior to GA_3 and/or 4-Cl-IAA (50 µL) or 0.1% Tween 80 application (SP and SPNS controls). The arrow indicates the time of hormone application. Data are means ± SE (n = 6)

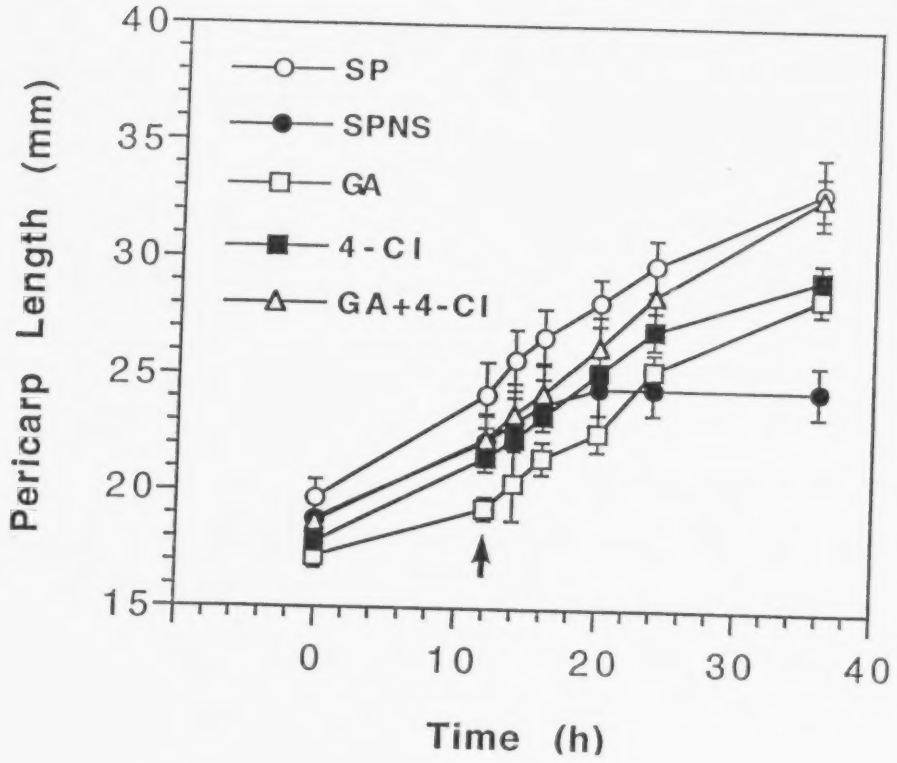

Pericarp Length (mm)
40
35
30
25
20
15
SP
SPNS
GA
4-Cl
GA+4-Cl
0
10
20
30
40
Time (h)

Figure 3.2. Time course of the accumulation of pea GA 20-oxidase mRNA in pericarp with seeds (SP), deseeded pericarp (SPNS), and deseeded pericarp treated with GA_3 (GA), 4-Cl-IAA (4-Cl), and GA_3 plus 4-Cl-IAA (GA+4-Cl) (A). Pericarps at 2 DAA (0 h) were split (SP) or split and deseeded. GA_3 and/or 4-Cl-IAA (50 μM) or 0.1% Tween 80 (SPNS control) were applied 12 h after deseeding. An actin probe was used as a loading control (B).

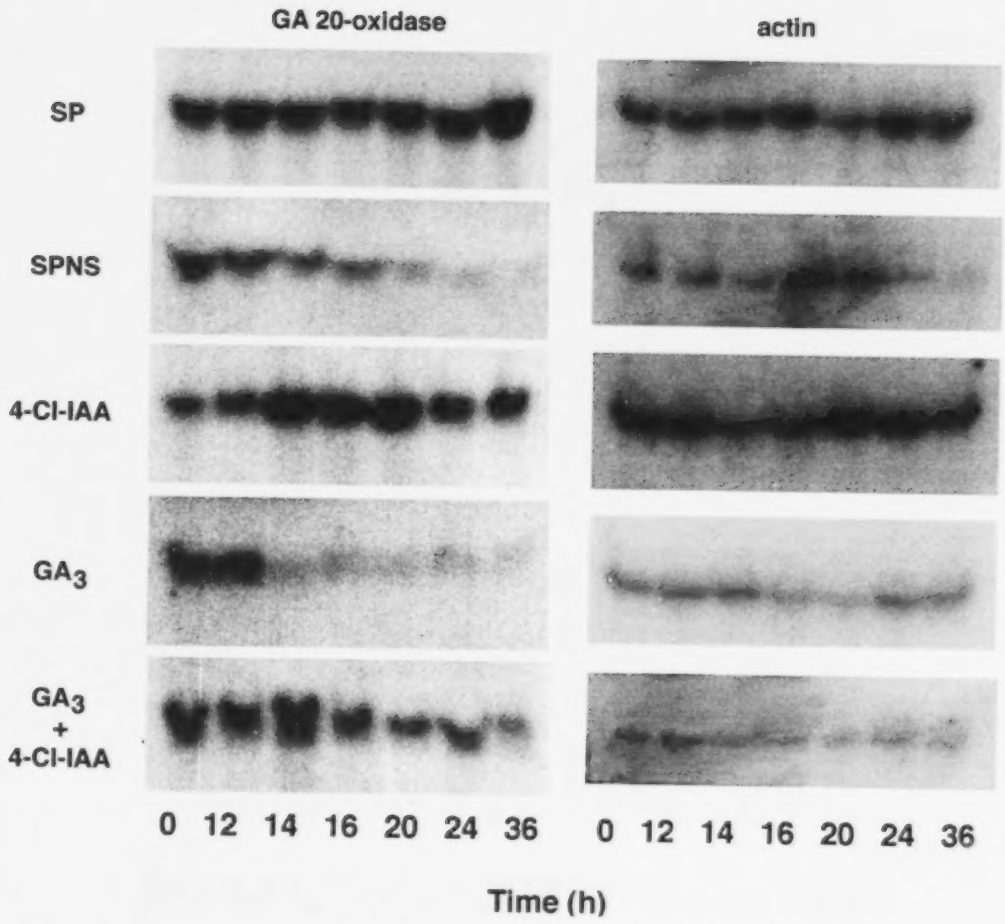
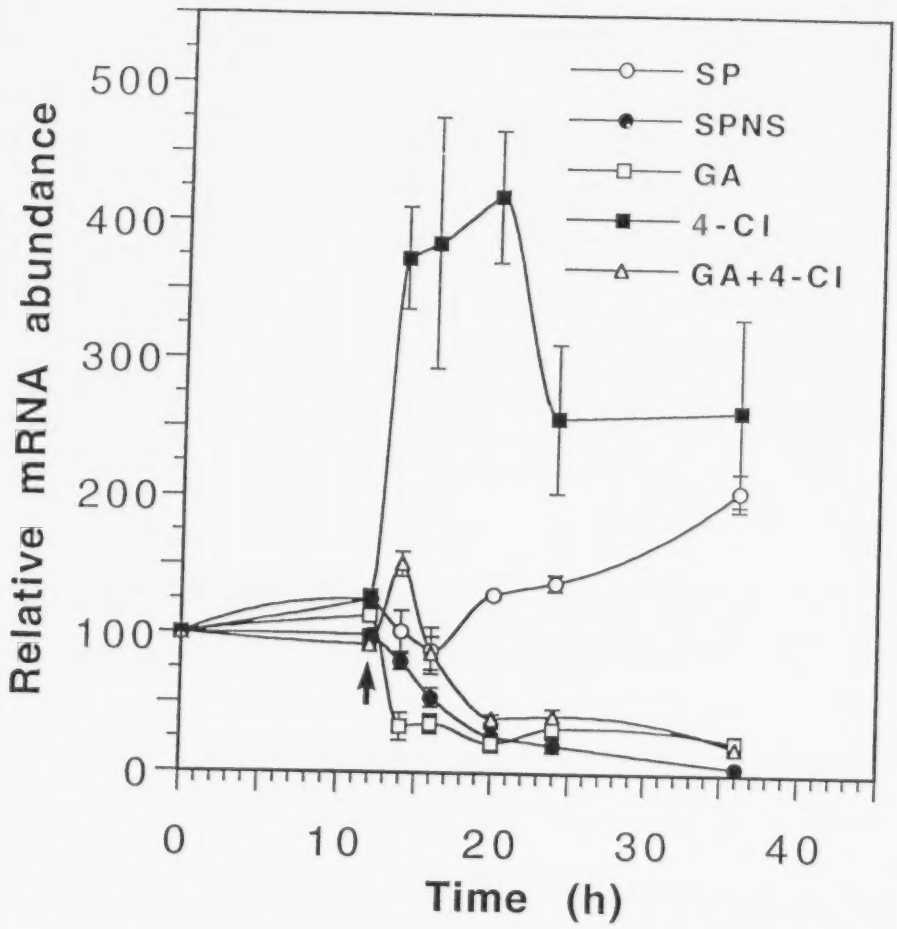

GA 20-oxidase
actin
SP
SPNS
4-Cl-IAA
GA3
GA3 + 4-Cl-IAA
0 12 14 16 20 24 36
0 12 14 16 20 24 36
Time (h)

Figure 3.3. Relative abundance of GA 20-oxidase transcripts in pea pericarp treated as described in Fig. 3.2. Hybridization signals were analyzed by scanning autoradiograms with an imaging densitometer. For the 2 DAA and the 12 h after deseeding samples, one extraction of 12-15 pericarps for each time period, was performed and these samples were used as standards for all blots. Hybridization signals were normalized to the value for pericarps at 2 DAA (0 h). The arrow indicates the time of hormone application. The data represent the average of two replicates ± SE, with one exception (for 4-Cl-IAA treatments n = 3).


Relative mRNA abundance
500
400
300
200
100
0
0
10
20
30
40
Time (h)
SP
SPNS
GA
4-CI
GA+4-CI

first 24 h after splitting the pericarp. After 36 h, GA 20-oxidase transcript levels had increased to a level about twice the level at 2 DAA. GA 20-oxidase mRNA levels in deseeded pericarp were similar to levels in pericarp with seeds during the first 12 h after seed removal, however after 12 h, GA 20-oxidase transcripts declined steadily to 5% of the original levels (2 DAA) after 36 h.

To allow sufficient time for the pericarp to become depleted of seed producal factors, that might affect the pericarp growth, hormones were applied to the pericarps 12 h after deseeding. When deseeded pericarp were treated with 4-Cl-IAA, a significant increase in GA 20-oxidase mRNA levels was detected within 2 h of hormone application. Transcript levels remained elevated for up to 8 h after 4-Cl-IAA application, and during this time period, were approximately 4 and 10 fold higher compared to levels in pericarp with seeds (SP) and deseeded pericarp (SPNS), respectively.

GA_3 significantly decreased GA 20-oxidase mRNA levels in deseeded pericarp within 2 h of application. Subsequently, GA 20-oxidase transcript levels remained considerably lower than levels in SP, but higher than levels in SPNS. 4-Cl-IAA application to GA_3-treated deseeded pericarps (GA_3+4-Cl-IAA treatment) delayed the decrease of GA 20-oxidase transcript levels from 2 to 8 h after hormone treatment.

DISCUSSION

We have shown that GA 20-oxidase mRNA levels in pea pericarp are maintained when seeds are present and decreased after seed removal to 5% of the original levels (2 DAA) by 36 h. These data demonstrate that seeds are required to maintain GA 20-oxidase mRNA levels for normal GA biosynthesis in the pericarp tissue. These findings are consistent with previous results from our laboratory that activity of the enzyme oxidizing GA_{19} to GA_{20} is maintained in pericarp with seeds and decreased to minimal levels in deseeded pericarp (Ozga et al., 1992; van Huizen et al., 1995). The pericarps are still

viable after seed removal as shown by their growth response to delayed hormonal application (GA_3 plus 4-Cl-IAA; van Huizen et al., 1995).

We have also shown that 4-Cl-IAA significantly increased levels of GA 20-oxidase transcripts in deseeded pericarp as compared to levels in pericarp with (SP) or without seeds (SPNS). These data agree with our previous findings that the *in vivo* activity of the GA_{19}-oxidizing enzyme increased in deseeded pericarp after 4-Cl-IAA treatment; metabolism of [^{14}C]GA_{19} to [^{14}C]GA_{20} and [^{14}C]GA_{29} was significantly higher in deseeded pericarp treated with 4-Cl-IAA than in pericarp with and without seeds (van Huizen et al., 1995). These results suggest that 4-Cl-IAA can substitute for the seeds in maintaining GA biosynthesis in the pericarp, at least in part by, stimulating or maintaining GA 20-oxidase transcript levels. GA 20-oxidase transcript levels were significantly higher in deseeded pericarp treated with 4-Cl-IAA compared to pericarp with seeds, even though the rate of pericarp growth of 4-Cl-IAA-treated pericarp was similar to pericarp with seeds. These data demonstrate that 4-Cl-IAA's effect on GA 20-oxidase mRNA abundance is not simply an indirect effect of 4-Cl-IAA on pericarp growth. The same conclusion was obtained in the [^{14}C]GA_{19} metabolism studies. [^{14}C]GA_{20} plus [^{14}C]GA_{29} levels were significantly higher in 4-Cl-IAA-treated deseeded pericarp than pericarp with seeds, but the rate of pericarp growth for 4-Cl-IAA-treated pericarp was lower than in pericarp with seeds.

Although the presence of 4-Cl-IAA in pea seeds has been known since the late 1960s (Marumo et al., 1968), the biological role of this endogenous halogenated auxin is not known. Reinecke et al. (1995) found that exogenous 4-Cl-IAA promoted pericarp growth, however IAA was ineffective in stimulating growth. Reinecke et al. (1995) also examined the effect of the position of the halogen (4-, 5-, 6- and 7-chloro- and fluoro-substituted IAA) on pericarp growth. In the pea pericarp assay, 4-Cl-IAA, and to a lesser extent 5-Cl-IAA promoted growth, while all other derivatives were inactive or inhibitory. In contrast, in pea stems and *Triticum* coleoptiles assays, IAA, 4-, 5-, 6-, and 7-Cl-IAA

and 5 F-IAA were all active, although maximum activity was observed at different concentrations (Katekar and Geissler, 1983; Hoffmann et al., 1952). In pea fruit, endogenous 4-Cl-IAA may have a specific role as a seed signal involved in the coordination of growth and development of the seed and surrounding pericarp tissue. One possible seed-regulatory mechanism in young pea fruit would be the export of 4-Cl-IAA by seeds to the pericarp where it stimulates GA biosynthesis.

In pea pericarp, down-regulation of GA 20-oxidase was observed within 2 h of GA_3 application. Hedden and Croker (1992) found that in maize seedlings bioactive GAs regulate their own biosynthesis through decreasing GA 20-oxidase activity. Phillips et al. (1995) found that application of GA_3 reduced expression of three GA 20-oxidase genes in flower shoots of the *ga1-2* mutant of *Arabidopsis*. In addition Xu et al. (1995) observed down-regulation of GA 20-oxidase mRNA by GA_4 in *Arabidopsis* leaves. These findings support the view that bioactive GAs may control their own synthesis through down regulation of the expression of the GA 20-oxidase genes. In our experiments the feedback inhibition of GA 20-oxidase expression by GA_3 was delayed by 4-Cl-IAA application. The mechanism by which 4-Cl-IAA elicits this response is not known. Further research is required to determine if 4-Cl-IAA and GA regulation of GA 20-oxidase expression is at the transcriptional and/or post-transcriptional level.

Our data suggest that GA 20-oxidase gene expression in pea pericarp is regulated by seeds, 4-Cl-IAA and GA_3. 4-Cl-IAA may be a seed factor, playing a role in the GA biosynthesis in pea pericarp by increasing levels and/or stability of GA 20-oxidase mRNA. The expression patterns of GA 20-oxidase in pea pericarp with seeds and deseeded pericarp treated with 4-Cl-IAA are very similar to the pattern of GA_{19} oxidase activity for these treatments obtained from our [^{14}C]GA_{19} metabolism studies

Stimulation of pea pericarp growth by 4-Cl-IAA appears to be, at least in part, by stimulating GA biosynthesis. However, experiments using the GA biosynthesis inhibitor paclobutrazol showed that 4-Cl-IAA also has a direct auxin effect on growth (Brenner and

Ozga, 1991). *In vivo* protein synthesis studies showed that the effects of GA_3 and 4-Cl-IAA on protein synthesis are not equivalent (van Huizen et al., 1996). We intend to investigate further the roles of GAs and auxins, and their interaction in pea fruit development at the molecular level.

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Chapter 4

Influence of Auxin and Gibberellin on *in Vivo* Protein Synthesis during Early Pea Fruit Growth

INTRODUCTION

The development of the seeds and the surrounding ovary (fruit) is closely integrated, but little is known about the biochemical and molecular interplay between them. In pea (*Pisum sativum* L.), normal pericarp growth requires the presence of seeds (Eeuwens and Schwabe, 1975). Removal of the seeds 2 to 3 DAA results in slowing of pericarp growth and subsequent abscission. The effect of seeds on fruit development, a process that involves cell division, cell enlargement, and cell differentiation, is assumed to involve plant hormones (Eeuwens and Schwabe, 1975; Gillaspy et al., 1993). Developing pea seeds contain GAs (biologically active GA_1 and GA_3; Garcia-Martinez et al., 1991) and auxins (4-Cl-IAA and IAA; Marumo et al., 1968; Ozga et al., 1993), and the effects of seeds on pericarp growth can be replaced by application of GAs (Eeuwens and Schwabe, 1975) or 4-Cl-IAA (Reinecke et al., 1995).

It has been assumed that the GAs biosynthesized by seeds are transported to the pericarp and regulate pericarp growth. However, an alternative hypothesis that seeds may promote pericarp growth by maintaining pericarp GA biosynthesis has been proposed (Sponsel, 1982). Results obtained using a split-pericarp system suggest that upon seed removal, a key step in the GA biosynthesis pathway is inhibited (conversion of GA_{19} to GA_{20}; Ozga et al., 1992). The auxin 4-Cl-IAA can substitute for the seeds in the stimulation of pericarp growth and the conversion of GA_{19} to GA_{20} (van Huizen et al., 1995). Experiments in which the GA biosynthesis inhibitor paclobutrazol was used suggested that a portion of 4-Cl-IAA stimulated growth is through its effect on GA biosynthesis (Brenner and Ozga, 1991). We have developed a working hypothesis that

envisions two roles for auxin in controlling fruit growth: (a) the export of 4-Cl-IAA from the seeds to the pericarp where it stimulates GA biosynthesis; and (b) a direct auxin effect of 4-Cl-IAA on pericarp growth. To test this hypothesis further and to broaden our understanding of hormone regulation of fruit development, critical analysis and timing of the molecular processes regulated by these hormonal signals is required.

Most of the molecular studies on the mechanism(s) of auxin- and GA-action have focused on auxin- or GA-induced cell elongation in vegetative tissues (coleoptiles, stems, hypocotyls, and epicotyls; Theologis, 1986; Chory et al., 1987). Auxin- and GA-mediated cell elongation is associated with changes in the expression of specific gene products (Theologis, 1986; Hagen, 1995; Chory et al., 1987; Shi et al., 1992; Phillips and Huttly, 1994). Several of these auxin- and GA-responsive genes have been characterized and the functions of the proteins they encode proposed (Phillips and Huttly, 1994; Abel et al., 1994).

Investigations of fruit development using molecular genetic techniques have concentrated mainly on fruit ripening (Gray et al., 1992), although changes in polypeptide patterns (Veluthambi and Poovaiah, 1984) and expression of several genes have been reported during early fruit development of tomato, kiwi fruit, strawberry, and pea (Narita and Gruissem, 1989; Salts et al., 1991; Ledger and Gardner, 1994; Reddy et al., 1990; Reddy and Poovaiah, 1990; Perez-Amador et al., 1995). Molecular studies investigating the regulatory role of auxin and GAs during early fruit development have focused on either auxin- or GA-regulated developmental processes (Veluthambi and Poovaiah, 1984; Reddy et al., 1990; Reddy and Poovaiah, 1990; Granell et al., 1992). Little is known about the relative roles of auxin and GA, or their interaction in young developing fruit. Our split-pod (pericarp) pea system offers the unique features of studying growth and development in a fruit in which the seed is accessible to manipulation, and which is responsive to both GA and auxin (4-Cl-IAA) *in planta* (Ozga and Reinecke, 1994; Reinecke et al., 1995). To begin a molecular analysis of the interaction of GAs and auxins during pea fruit

development, we have utilized *in vivo* protein labeling with [^{35}S]Met coupled with two-dimensional PAGE to obtain a profile of hormonal and seed-induced polypeptide changes during the early fruit development. We report here on the patterns of protein expression associated with 4-Cl-IAA, GA_3, and seed-induced pea pericarp growth.

MATERIAL AND METHODS

Plant Material and Treatments

Plants of *Pisum sativum* L., line I_3 (Alaska-type) were grown as previously described (van Huizen et al., 1995). One fruit per plant (at the third to fifth flowering node) was treated, and subsequent flowers were removed as they developed. Terminal apical meristems of plants were intact and the pericarp remained attached to the plant during the entire experiment. To remove the seeds, a split-pericarp technique was used as described by Ozga et al. (1992). Briefly, pericarps of 2 DAA (15-20 mm) ovaries (pericarp + seeds) were split down the dorsal suture, either without disturbing the seeds (SP treatment) or the seeds were removed immediately (SPNS treatment). Eight or 14 h later, the SP and SPNS pericarp were treated with 10 µCi of L-[^{35}S]Met (>1000 Ci/mmol; Amersham, Canada) in 10 µL of 0.1% Tween 80. Two hours prior to [^{35}S]Met application (14 h treatments only), deseeded pericarps were treated with GA_3 and/or 4-Cl-IAA (30 µL, 50 µM in 0.1% Tween 80) or 0.1% Tween 80 alone (SP and SPNS controls). In some treatments, paclobutrazol, an inhibitor of GA biosynthesis, was applied to the pericarp (30 uL, 50 µM in 0.1% Tween 80) immediately after deseeding. All treatments were applied to the inside surface (endocarp) of the pericarp. After a 4 h incubation period with L-[^{35}S]Met, the pericarp tissues (seeds were removed if present) were harvested 12 h (SP and SPNS) or 18 h (SP, SPNS, and hormone treatments) after pericarp splitting and frozen immediately in liquid N_2 and stored at -80^{o}C until extracted.

High humidity was maintained by enclosing the fruits in clear plastic bags throughout the duration of the experiment.

Extraction of Total Protein

Total protein was extracted as follows. Frozen pericarp (two per sample) were homogenized to a fine powder in a mortar containing liquid N_2. When the temperature reached approximately 0°C, 0.5 mL of extraction buffer (50 mM Tris HCl, pH 7.5, 2% SDS, 150 mM DTT and 1 mM PMSF) was added. The extract was centrifuged at 10,000*g* for 10 min and the clear supernatant was heated at 100°C for 3 min and cooled on ice. Proteins were precipitated by adding cold acetone (8X volume of supernatant) and kept at -20°C for at least 1 h. The acetone precipitates were pelleted by centrifugation at 10,000*g* for 10 min at 4°C. After the acetone was removed, the pellet was air-dried and resuspended in a buffer containing 9.5 M urea, 5 mM K_2CO_3, 4% CHAPS (Sigma Chem. Co, St. Louis, MO), 5% ampholytes (2 parts pH 4.0-5.0, 2 parts pH 5.0-7.0 and 1 part pH 3.0-10.0, Bio-Rad). The insoluble material was removed by centrifugation at 10,000*g* for 30 min. An aliquot containing 400,000 TCA-precipitable dpms was loaded on each IEF gel (Gel electrophoresis cell model 155, Bio-Rad). Total protein loaded per IEF gel was approximately 80 µg.

Two Dimensional Gel Electrophoresis

Two dimensional PAGE was carried out as described by Xin and Li (1993) with the following modification. The final combined concentration of ampholytes in the IEF gel solution was 5% (2 parts pH 4.0-5.0, 2 parts pH 5.0-7.0 and 1 part pH 3.0-10.0). Second dimension electrophoresis was performed as described by Laemmli (1970) using 4% acrylamide for the stacking gel and 14% acrylamide for the running gel. Gels were run

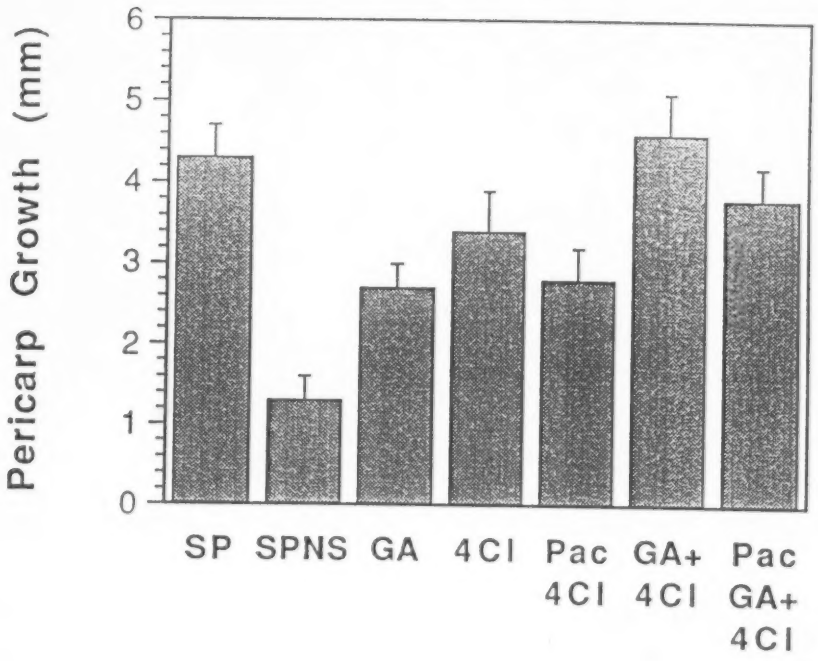
on a Protean II xi cell (Bio-rad) at a constant current of 25 mA/gel at 18°C for 3.5 h. After overnight fixation in methanol:acetic acid:H2O (40:10:50[v/v]), gels were infiltrated with 2,5-diphenoloxazole as described by Laskey and Mills (1975). Gels were dried onto Whatmann 3 MM paper and exposed to preflashed Kodak X-Omat AR x-ray film at -80°C for approximately 10 d. All treatments were repeated a minimum of three times and the reproducible changes were analyzed by scanning the film with an imaging densitometer (Bio-Rad). For integration of X-ray film images, Bio-Rad Molecular Analyst/PC Image Analysis software (version 1.3) was used. The area of each individual protein spot image was approximated best by enclosing it within an ellipse. The total digital signal or OD within an ellipse was determined, and a background OD value was subtracted from the total OD of the protein spot image to remove values contributed from background noise. The percentage of relative abundance of specific radiolabeled polypeptides was determined by comparing the OD of the specific protein with a standard protein whose labeling pattern was consistent between treatments (the same standard protein was used to normalize all samples) using the following formula:

$$\text{Relative abundance (\%)} = \frac{\text{Total OD (specific protein) - background OD}}{\text{Total OD (standard protein) - background OD}} \times 100$$

RESULTS

Pericarp growth (length) was similar in pericarp with and without seeds 8 h (2.3 ± 0.3 mm) or 12 h after pericarp splitting (prior to hormone application; 3.5 ± 0.3 mm). However, during the 6 h hormone incubation period, pericarp growth differed significantly among treatments (F test; $P < 0.0001$). SPNS growth was significantly reduced compared

Figure 4.1. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with GA_3 (GA), 4-Cl-IAA (4-Cl), paclobutrazol plus 4-Cl-IAA (Pac 4-Cl), GA_3 plus 4-Cl-IAA (GA + 4-Cl), and paclobutrazol plus GA_3 plus 4-Cl-IAA (Pac GA + 4-Cl) on pea pericarp growth. Two DAA pericarps were deseeded 12 h prior to GA_3 and/or 4-Cl-IAA (50 μM) or 0.1% Tween 80 application (SP and SPNS controls). Paclobutrazol was applied to the pericarp (50 μM) immediately after deseeding for the paclobutrazol treatments. Pericarp growth = final length (6 h after hormone treatment) minus initial length at time of hormone application; data are means ± SE (n = 6)


Pericarp Growth (mm)
0
1
2
3
4
5
6
SP
SPNS
GA
4Cl
Pac 4Cl
GA+ 4Cl
Pac GA+ 4Cl

with pericarp with seeds (LSD, P = 0.5; Fig. 4.1). GA_3 and 4-Cl-IAA significantly stimulated growth of deseeded pericarps compared with the deseeded control (SPNS; LSD, P = 0.5). The combination of GA_3 plus 4-Cl-IAA had additive effects on growth of deseeded pericarps, resulting in growth similar to that of pericarps with seeds. The addition of paclobutrazol had only a minor effect on the growth response to 4-Cl-IAA with or without GA_3.

Analysis of about 250 radiolabeled protein spots revealed that the most significant and reproducible changes were observed in polypeptides with M_rs between 20 and 60 (Figs.4.2 and 4.3). The polypeptide changes were grouped into 6 classes: 4-Cl-IAA induced, 4-Cl-IAA repressed, GA_3 induced, GA_3 and 4-Cl-IAA induced, GA_3 and 4-Cl-IAA repressed, and seed induced (Table 4.1). The greatest difference in *in vivo*-labeled polypeptide patterns occurred between pericarp with seeds and deseeded pericarp (SPNS) treatments (Figs. 4.2 and 4.3, A and B). Seed removal 8 h prior to [^{35}S]Met application resulted in the appearance of or increase in 6 (3, 4, 7, 8, 9, 11) and the disappearance or decrease in 9 (A-I) labeled polypeptides (Fig. 4.2). The pattern of labeled polypeptides in pericarp deseeded 14 h prior to [^{35}S]Met application was similar to, but not identical with, the 8 h treatment pattern (Fig. 4.2 and 4.3, A and B). In pericarp deseeded 14 h prior to [^{35}S]Met application, polypeptide 9 had increased and 5 additional labeled polypeptides had appeared or increased (1, 2, 5, 6, 10; Fig. 4.3B) in comparison with pericarp deseeded 8 h prior to [^{35}S]Met application (Fig. 4.2).

Treatment of deseeded pericarps with 4-Cl-IAA or 4-Cl-IAA plus GA_3 inhibited accumulation of 11 polypeptides (1-11) induced by seed removal to levels similar to those observed in pericarp with seeds (Fig. 4.3; abundance of selected polypeptides, Fig. 4.4 ,E and F). Application of GA_3 alone to deseeded pericarp suppressed only 8 of these 11 polypeptides (polypeptides 1, 2, 3, 4, 6, 7, 8, 11). The net synthesis of polypeptides specifically inhibited by 4-Cl-IAA (5, 9, 10; Fig. 4.4E) was 3 to 11 times higher in the deseeded (SPNS, 14 h treatment) and GA_3-treated deseeded pericarp than in pericarp

Figure 4.2. Fluorographs of [^{35}S]Met-labeled polypeptides from pericarps 2 DAA resolved by electrophoresis on two-dimensional gels. Pericarps were split (SP; A) or split and deseeded (SPNS; B) 8 h prior to [^{35}S]Met application. Letters (A-I) adjacent to arrows designate polypeptides enhanced by hormone and/or seed treatments. Numbers (1-11) adjacent to circles designate polypeptides repressed by hormone and/or seed treatment. The standard protein used to normalize the abundance of specific radiolabeled polypeptides is enclosed within a square on each fluorograph. Each treatment was repeated at least 3 times and representative fluorographs are shown.

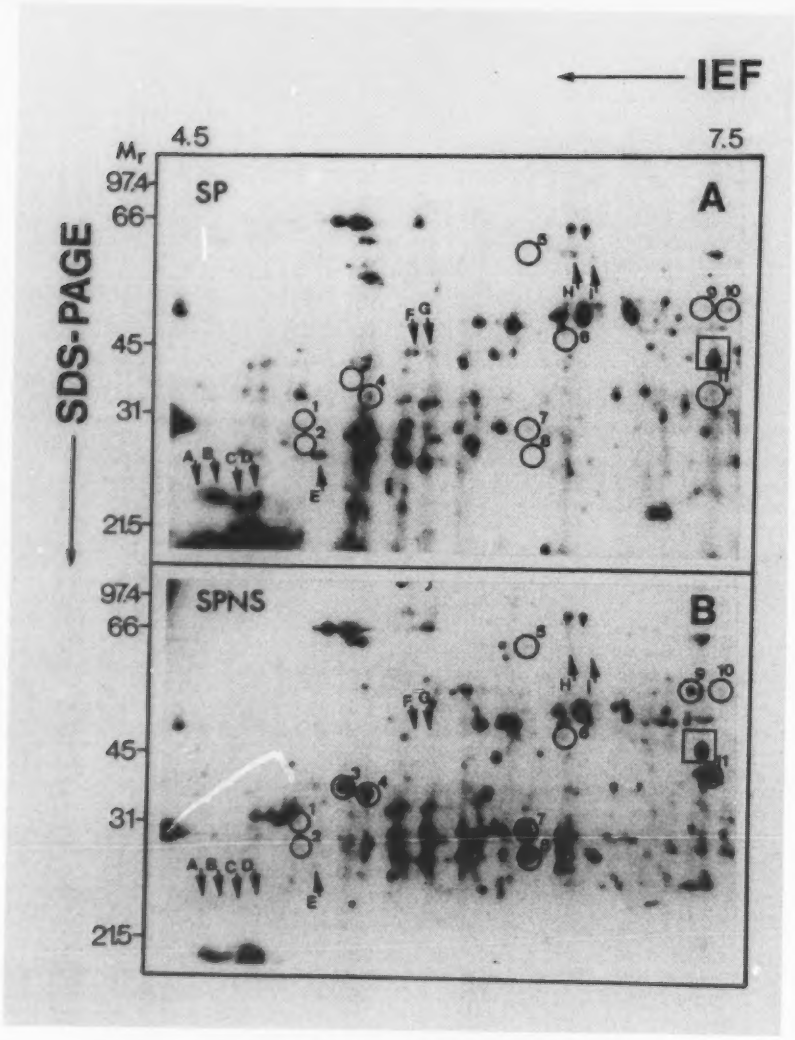
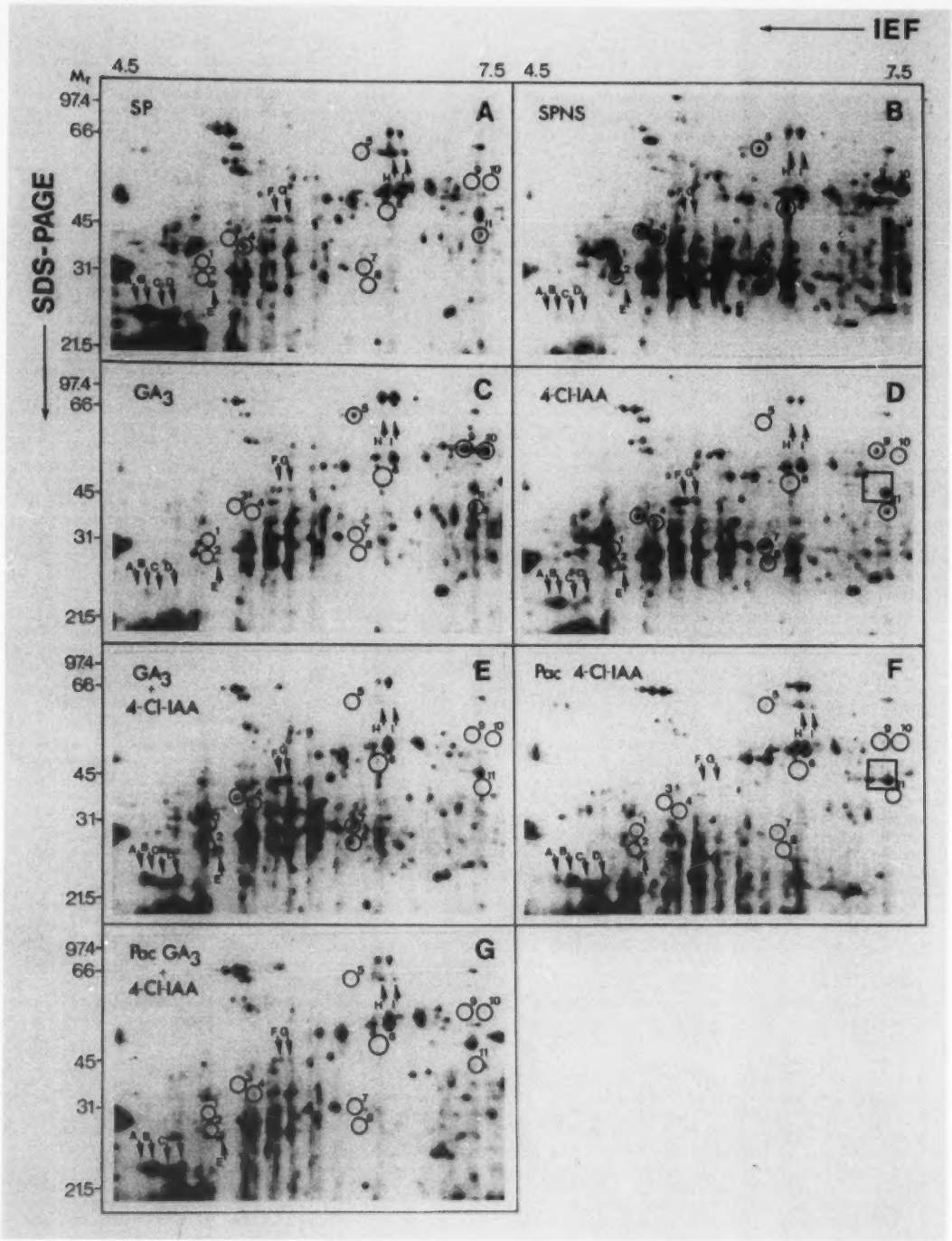

IEF
4.5
7.5
M_r
97.4
66
45
31
21.5
SDS-PAGE
SP
A
SPNS
B

Figure 4.3. Fluorographs of [^{35}S]Met-labeled polypeptides from 2 DAA pericarps resolved by electrophoresis on two-dimensional gels. Pericarps were split (SP; A) or split and deseeded (B, C, D, E, F, G) 14 h prior to [^{35}S]Met application. Two h prior to [^{35}S]Met application, pericarps were treated with 0.1% Tween 80 (controls: SP, A; and SPNS, B) or 50 μM solutions of GA_3 (C), 4-Cl-IAA (D), GA_3 plus 4-Cl-IAA (E), paclobutrazol plus 4-Cl-IAA (F), paclobutrazol plus GA_3 plus 4-Cl-IAA (G). Polypeptide nomenclature is the same as in Figure 4.2. Polypeptide nomenclature and standard protein designations are the same as in Figure 4.2. Each treatment was repeated at least 3 times and representative fluorographs are shown.


IEF
SDS-PAGE
Mr
4.5
7.5
97.4
66
45
31
21.5
SP
A
SPNS
B
GA3
C
4-Cl-IAA
D
GA3
+
4-Cl-IAA
E
Poc 4-Cl-IAA
F
Poc GA3
+
4-Cl-IAA
G

treated with 4-Cl-IAA (Fig. 4.4E). Polypeptides 5, 9, and 10 also were in low abundance in pericarp with seeds (SP).

The abundance of 4 labeled polypeptides (A, B, C, D) increased in deseeded pericarps when treated with 4-Cl-IAA, but not with GA_3 alone (Fig. 4.3). The levels of polypeptide B in 4-Cl-IAA-treated tissue were similar to those in pericarp with seeds, and approximately 6 to 10 times higher than those in GA_3-treated deseeded pericarp and SPNS controls (Fig. 4.4A).

Because 4-Cl-IAA has the capacity to stimulate a key step in the GA biosynthesis pathway, paclobutrazol was applied to resolve the selective contribution of 4-Cl-IAA and GA_3 on protein synthesis in early pea fruit growth. Paclobutrazol inhibited accumulation of polypeptides F and G in tissue treated with 4-Cl-IAA (Pac 4Cl), but not in pericarp treated with both 4-Cl-IAA and GA_3 (Pac GA_3 + 4-Cl; Fig.4.4B). Polypeptides F and G were 6 to 15 more abundant in the GA_3-, 4-Cl-IAA-, and GA_3 plus 4-Cl-IAA-treated deseeded pericarp than in the SPNS control. Polypeptides F and G were also detected in pericarp with seeds (SP, 14 h treatment) at levels 6 and 9 times higher, respectively, than in deseeded pericarp (SPNS). These data suggest that polypeptides F and G are regulated by GAs.

One polypeptide (E) was induced in both GA_3- and 4-Cl-IAA-treated deseeded pericarp (Fig. 4.3). Its levels were approximately 4 to 6 times higher in the GA_3-, 4-Cl-IAA- and GA_3 plus 4-Cl-IAA-treated deseeded pericarp than in the SPNS (Fig. 4.4C). Polypeptide E was also present in pericarp with seeds (SP) at a level similar to that found in hormone-treated pericarp.

Among the polypeptides analyzed, two were more abundant (H, 4-13 times; I, 4-11 times) in pericarp with seeds (SP) than in deseeded pericarp regardless of treatment (Fig. 4.3). Labelling of polypeptide H in the SP treatment doubled from 8 h to the 14 h treatment period (Fig. 4.4D).

Figure 4.4. Relative abundance of six classes of [^{35}S]Met-labeled polypeptides in pea pericarp; 4-Cl-IAA induced (A); GA_3 induced (B); GA_3 & 4-Cl-IAA induced (C); Seed specific induced (D); 4-Cl-IAA repressed (E); GA_3 plus 4-Cl-IAA repressed (F). Quantitation of fluorographs was accomplished as described in "Material and Methods" section. The results represent the average of 3 replicates ± SE, with one exception (for Pac 4Cl treatment n = 2)

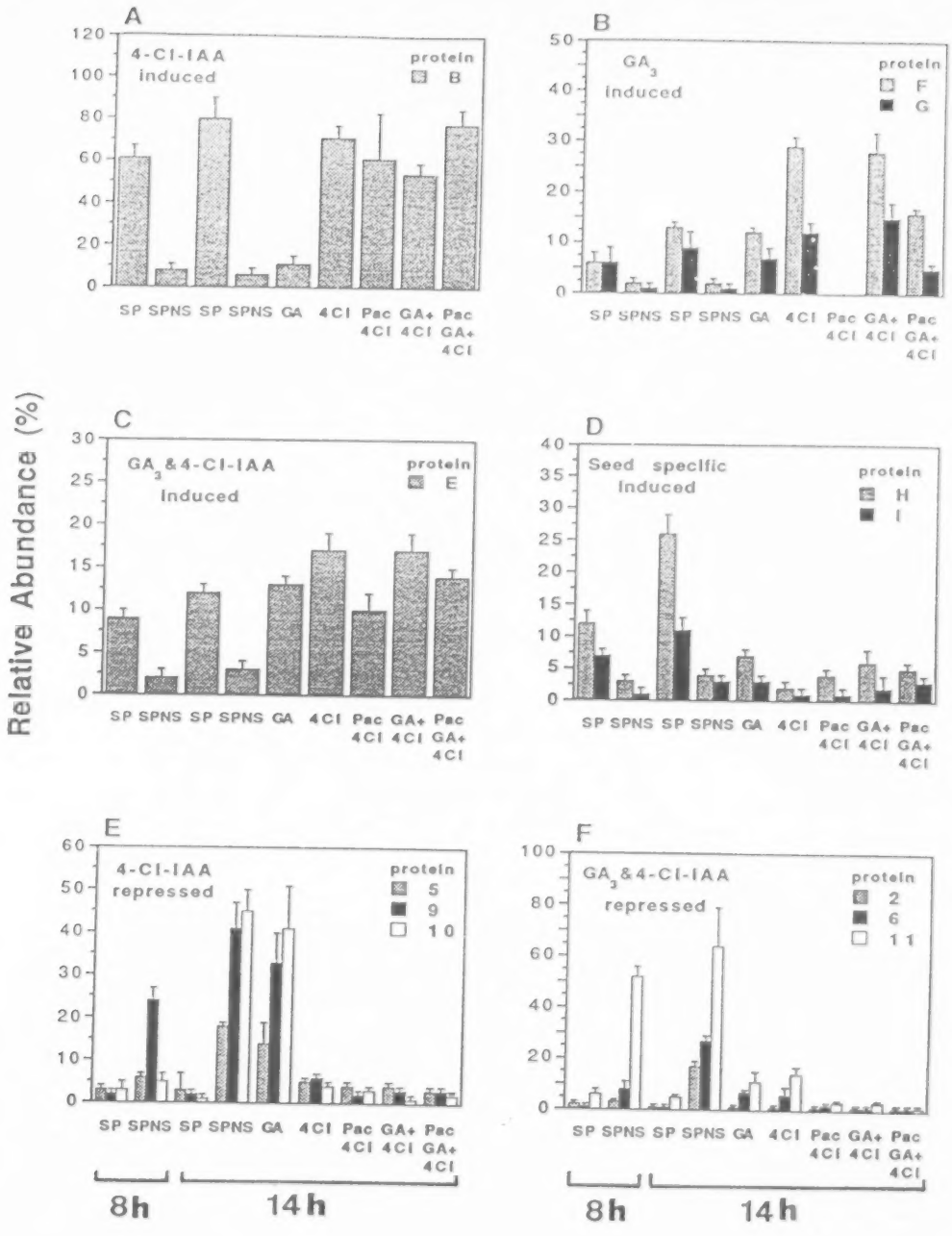

A
4-Cl-IAA
induced
protein
B
B
GA3
induced
protein
F
G
C
GA3&4-Cl-IAA
Induced
protein
E
D
Seed specific
Induced
protein
H
I
E
4-Cl-IAA
repressed
protein
5
9
10
F
GA3&4-Cl-IAA
repressed
protein
2
6
11
Relative Abundance (%)
SP SPNS SP SPNS GA 4Cl Pac 4Cl GA+ 4Cl Pac GA+ 4Cl
8h
14h

Table 4.1. Summary of the major *in vivo* labeled polypeptide changes in pea pericarp 2 DAA as a result of GA_3, 4-Cl-IAA, or seed treatments

Polypeptide Induction			Polypeptide Repression		
Inducer	ID[a]	Mr	Repressor	ID	Mr
4-Cl-IAA	A	24.7	4-Cl-IAA	5	62.8
	B	24.3		9	54.5
	C	23.7		10	53.6
	D	24.9			
GA_3 and 4-Cl-IAA	E	28.5	GA_3 and 4-Cl-IAA	1	32.3
				2	29.4
GA_3	F	45.0		3	38.4
	G	45.4		4	37.2
				6	47.2
Seed	H	63.8		7	30.8
	I	63.8		8	28.5
				11	39.7

[a]Polypeptide nomenclature

Since the combination of 4-Cl-IAA plus GA_3 stimulates pericarp growth more than 4-Cl-IAA or GA_3 alone (Ozga and Reinecke, 1994), we compared protein profiles of 4-Cl-IAA plus GA_3-, 4-Cl-IAA-, and GA_3-treated deseeded pericarp. 4-Cl-IAA plus GA_3 and 4-Cl-IAA alone were very similar in enhancing and suppressing the synthesis of proteins in pericarp tissue (Fig. 4.3, D and E, 4, A, C, E and F).

DISCUSSION

Our previous growth and metabolism studies showed that 4-Cl-IAA, an endogenous pea auxin, can stimulate pericarp growth and GA biosynthesis in the pericarp, specifically the conversion of GA_{19} to GA_{20}. In this study, the effect of GA, auxin, and seeds on *in vivo*-protein synthesis in pea pericarp was used to assess, at the protein level, the relative contribution of these hormones to pea fruit development and to evaluate the extent to which these hormones can mimic the seed in this process. Our data reveal that seed removal greatly modifies *in vivo* labeled protein patterns. As soon as 12 h after seed removal, 6 labeled polypeptides appeared or increased (deseeded 8 h prior to [^{35}S]Met application; Fig. 4.2). An additional 5 polypeptides appeared or increased 18 h after pericarp deseeding (deseeded 14 h prior to [^{35}S]Met application; Fig. 4.3). These pericarps are still viable after seed removal as shown by their growth response to delayed hormonal application (GA_3 plus 4-Cl-IAA; van Huizen et al., 1995). During early pea fruit development, seed factors may normally suppress the synthesis of this class of polypeptides.

Hormonal effects on protein synthesis in deseeded pericarp were detected within 6 h of hormone application. Accumulation of all 11 polypeptides specific to deseeded pericarp was inhibited by 4-Cl-IAA application to levels similar to that in pericarp with seeds (Fig. 4.3; 4.4, E and F). Application of GA_3 to deseeded pericarp suppressed only 8 of these 11 polypeptides (polypeptides 1, 2, 3, 4, 6, 7, 8, 11). Since growth of the GA_3-

and 4-Cl-IAA-treated deseeded pericarp was similar (Fig. 4.1), polypeptides 5, 9, and 10 appear to be specifically auxin-repressed and not repressed by GA_3 or indirectly by growth. Auxin- or GA-repressed polypeptide synthesis has been characterized in various plant systems (Theologis, 1986; Chory et al., 1987). Veluthambi and Poovaiah (1984) found that removal of the achenes, which inhibits strawberry fruit growth, resulted in the appearance of two polypeptides of 52 and 57 kD. Exogenous naphthalene acetic acid (NAA), which induces growth in receptacles from which achenes have been removed, prevented the appearance of these two polypeptides. The authors suggested that these proteins may have an inhibitory role in strawberry fruit development. This may also be the case for polypeptides 1, 2, 3, 4, 6, 7, 8, 11 observed in pea fruit. However we have observed an additional class of proteins (polypeptides 5, 9, 10) that appear to be specifically repressed by auxin, but not indirectly by pericarp growth.

In pea fruit, endogenous 4-Cl-IAA may act as a seed signal stimulating GA biosynthesis in pericarp tissue (Ozga et al., 1992; van Huizen et al., 1995). Paclobutrazol was applied to resolve the effects of 4-Cl-IAA and GA_3 on protein synthesis in early pea fruit growth. Two proteins (F, G; Fig. 4.4B) were identified as GA_3 induced because they were not detected in deseeded pericarp treated with paclobutrazol plus 4-Cl-IAA, but were present when GA_3 was added.

4-Cl-IAA also increased labeling or appearance of 4 proteins (A-D, Fig. 4.3, Table 4.1) which were of low abundance or not detected in deseeded pericarp treated with GA_3 alone. These 4 proteins were also present in pericarp with seeds, therefore, endogenous 4-Cl-IAA may induce synthesis of proteins required for pericarp growth. Veluthambi and Poovaiah (1984) found that NAA applied to achene-removed strawberry fruit stimulated growth and accumulation of 3 polypeptides. By simultaneously monitoring auxin- and GA-induced protein synthesis, we have observed a class of proteins (polypeptides A-D) that appear to be specifically induced by auxin, but not by GA_3-induced pericarp growth.

These results support our hypothesis that 4-Cl-IAA also has a direct auxin-mediated effect on pericarp growth.

Two polypeptides (H, I; Fig. 4.4D) were abundant in the pericarp only when the seeds were present; neither GA_3 nor 4-Cl-IAA induced their synthesis. One possible explanation is that seed factors other than 4-Cl-IAA or GA_3 may be involved in regulating protein synthesis in pea pericarp. Alternatively these proteins may be induced by 4-Cl-IAA and/or GA_3, but after a longer incubation time.

Pea fruit growth is a carefully regulated process in time and space, beginning with pollination and fertilization, followed by pericarp growth, and, lastly, with seed growth and maturation. GAs and auxins are natural constituents of pea fruit which likely play a role in pea fruit growth and development. Within 12 h after deseeding, pericarp tissue responds to seed removal by synthesizing, inhibiting synthesis, or modifying specific proteins (Fig. 4.2). Application of 4-Cl-IAA and GA_3 to pericarp 12 h after deseeding reverses this process (Figs. 4.3 and 4.4). Similar patterns of protein expression were associated with both hormone treatments; however, polypeptide patterns unique to GA_3 or 4-Cl-IAA also indicate that GA_3 and 4-Cl-IAA effects on this process are not equivalent. In general, application of 4-Cl-IAA plus GA_3 replaced the seed effect on protein synthesis in the pericarp, supporting our hypothesis that both hormones are involved in pea pericarp development. The results presented are unique in that both GA_3 and 4-Cl-IAA effects on protein synthesis were monitored simultaneously, in a tissue system that naturally contains 4-Cl-IAA and GA_3. These data suggest that at least part of seed regulation of *in vivo* protein synthesis in the pericarp tissue is directly through hormonal signals from the seeds, other seed factors, or events that modify hormonal levels in the pericarp.
Using the pea split-pericarp growth system we intend to examine further the relative roles of auxins and GAs on pericarp development at the molecular level.

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Chapter 5

Molecular Characterization of Cell Division and Cell Elongation during Early Pea Pericarp Growth

INTRODUCTION

Fruit development involves a complex interaction of molecular, biochemical, and structural changes that transform a fertilized ovary into a mature fruit. Fruit development in pea has been well characterized physiologically and biochemically (Hebblethwaite et al., 1985; Sutcliffe and Pate, 1977) and represents a useful system to learn more about complex regulatory mechanisms that control the division, growth and differentiation of plant cells. However, little information is available on the molecular aspects of fruit development, and how this development is coordinated with seed formation. Pea fruit development provides a useful model system to define the signals produced by the developing seeds directing cell activities in the surrounding tissues. Fruit set and sustained pod elongation in pea are normally dependent on the presence of seeds. It is likely that the developing seeds produce signal molecules that regulate cell division and cell expansion of the surrounding fruit tissues. GAs (biologically active GA_1 and GA_3; Garcia-Martinez et al., 1991) and auxins (4-Cl-IAA and IAA; Marumo et al., 1968; Ozga et al., 1993) are natural constituents of pea seeds and pericarps and are likely candidates for such signal molecules. In pea, it has been assumed that GAs biosynthesized by the seeds are transported to the pericarp and regulate pericarp growth (Garcia-Martinez et al., 1991). However, an alternative hypothesis that seeds may promote pericarp growth by maintaining pericarp GA biosynthesis has been proposed (Sponsel, 1982). Results obtained using a split-pericarp system suggest that upon seed removal, a key step in the GA biosynthesis pathway is inhibited (conversion of GA_{19} to GA_{20}; Ozga et al., 1992). The auxin 4-Cl-IAA can substitute for the seeds in the

stimulation of pericarp growth and the conversion of GA_{19} to GA_{20} (van Huizen et al., 1995). 4-Cl-IAA may be a seed factor that stimulates GA biosynthesis in the pericarp, but also has an independent auxin effect on pericarp growth (Reinecke et al., 1995; van Huizen et al., 1995; van Huizen et al., 1996).

Auxin and GAs have long been acknowledged as regulators of cell division and elongation (Davies, 1995; Evans, 1984). The objectives of this study were to use molecular markers combined with histological studies to characterize mitotic activity and cell expansion during early pea pericarp development and during GA- and 4-Cl-IAA-stimulated pericarp growth.

The availability of plant cell cycle-dependent genes provide a means for examining the pattern of mitotic activity during early fruit development and the influence of plant growth regulators on this process. The regulation of replication-dependent histone expression has been extensively studied and reviewed (Osley, 1991). Tanimoto et al. (1993) found that histone H2A mRNA transiently accumulates in apical meristems in pea root tips during a period of the cell cycle that mostly overlaps the S phase. In tomato, Koning et al. (1991) found that the steady-state histone H2A was abundant in cycling cells like apices and early developing fruit and very low in mature tissue. Therefore, expression of this histone is replication dependent and accumulation of its mRNA may be useful as a marker for cell division as long as endoduplication in the tissue of interest is minimal (Koning et al., 1991).

Before the cell enlargement phase, cells in the developing pea fruits are small, and tightly compressed. As cells enlarge, the primary cell wall and the cytoplasmic layer become relatively thinner, and vacuoles occupy a greater proportion of the cell volume (Vercher et al., 1984). γ–TIP, a tonoplast intrinsic protein, is a member of a small gene family (TIPS), which are differentially expressed in organs or as a result of specific signals (Ludevid et al., 1992). γ–TIPs are capable of forming transmembrane channels that allow the passive transfer of water (Maurel et al., 1993). In *Arabidopsis*, γ–TIP is expressed

primarily at the time when large central vacuoles are being formed during cell enlargement (Ludevid et al., 1992). Since γ–TIP expression is correlated with regions of cell expansion, γ-TIP mRNA accumulation may be a good marker for cell elongation.

Application of GA_3 to the *Arabidopis ga1* mutant, which has very low levels of endogenous active GAs, induced stem elongation and γ–TIP expression (Phillips and Huttly, 1994). GA_3 may stimulate γ–TIP expression directly, or indirectly by stimulating cell expansion. Our split-pod pea system offers the unique features of studying γ–TIP expression in elongating pericarp tissue, which is responsive to both GAs and auxin (4-Cl-IAA).

In this study, histone H2A and γ–TIP genes were used as molecular probes to follow the pattern of cell division and cell enlargement at different stages of pea fruit development and in GA_3- and/or 4-Cl-IAA treated-pericarp. We also examined the structural features associated with GA_3- and 4-Cl-IAA-stimulated pericarp growth. Our results suggest that both GA_3 and 4-Cl-IAA are required for pea pericarp growth.

MATERIAL AND METHODS

Plant Material and Treatments

Plants of *Pisum sativum* L., line I_3 (Alaska-type) were grown as previously described (van Huizen et al., 1995). One fruit per plant (at the third to fifth flowering node) was used per treatment, and subsequent flowers were removed as they developed. Terminal apical meristems of plants were intact and the pericarp remained attached to the plant during the entire experiment. To remove the seeds, a split pod technique was used as described by Ozga et al. (1992). Briefly, pericarps of 2 DAA (15-22 mm) ovaries (pericarp + seeds) were left intact (intact treatment) or split down the dorsal suture, either without disturbing the seeds (SP treatment) or the seeds were removed immediately (SPNS treatment). GA_3 and/or 4-Cl-IAA (50 μM in 0.1% Tween 80) were applied immediately

after deseeding to the inner pericarp wall. Control treatments (SP and SPNS) were treated with 0.1% Tween 80. In some treatments, paclobutrazol, an inhibitor of GA biosynthesis, was applied immediately after deseeding to the pericarp (30 µL, 50 µM in 0.1% Tween 80). High humidity was maintained by enclosing the pericarps in clear plastic bags throughout the duration of the experiment.

RNA Isolation and Northern Analysis

For each sample, 2-3 pods were ground in liquid N_2 and a 0.3 - 0.5 g subsample was used for RNA extraction. For the 2 DAA samples, approximately 15 pericarps were pooled for RNA extraction and this sample was used as a standard for all blots with one exception (γ-TIP-paclobutrazol experiment). Total RNA was extracted following the method of Chomczynski and Sacchi (1987) with 2 additional chloroform extractions after the first chloroform extraction to remove polysaccharides. For northern analysis, the total RNA samples (10 µg) were denatured in 2.2 M formaldehyde/50% formamide, fractionated on a 1.2% agarose/2.2 M formaldehyde gel using a 20 mM MOPS buffer (pH 7.0; Maniatis et al., 1982) and transferred to Nitroplus membranes (MSI, Westborough, USA) with 10X SSC. Equal loading and RNA integrity were ascertained by ethidium bromide staining of ribosomal RNA bands prior to transfer. Membranes were baked for 2 h at 80°C under vacuum. The [^{32}P]dATP random-primed cDNA probes were synthesized using the random primers DNA labeling system (GIBCO-BRL) according to the manufacturers instructions. Filters were prehybridized, and then hybridized with the labeled probe at 65°C in a solution containing 6X SSPE, 0.5% SDS, 5X Denhardt's solution (1% Ficoll 400, 1% PVP, 1% BSA), and 100 µg mL^{-1} t-RNA for 18 h. Membranes were washed three times for 20 min at room temperature in 2X SSPE, 0.1% SDS, and once in 0.1% SCC, 0.1% SDS at 65°C, and placed at -70°C with Kodak X-Omat AR film. The northern probes were a 0.6 kb *Eco* RI fragment of histone H2A

cDNA from pea (Koning et al., 1991), and a 1.4 kb *Bam* HI-*HindIII* fragment of γ–TIP cDNA from *Arabidopsis* (Hofte et al., 1992). Some blots were stripped in H_2O at 100°C for 5 min and reprobed with an *Arabidopsis* ubiquitin random-primed cDNA probe (clone UBQ4 from Dr. Vierstra, University of Wisconsin, Madison). Probe labeling, prehybridization and hybridization were as described above. The relative abundances of H2A and γ–TIP mRNA were estimated by scanning the autoradiogram with an imaging densitometer (BIO-RAD). The value at 2 DAA on each autoradiograph was designated 100% and all other signals were calculated relative to that sample.

Light microscopy

Light microscopy was conducted on transverse sections of the midregion of the pericarp wall. Fixation was overnight at 20°C in 3% glutaraldehyde fixative in 0.1 M phosphate buffer. After fixation, tissue segments were dehydrated through a graded series of ethanol (at 30 minute intervals for each 15% increment in ethanol). Dehydrated tissues were infiltrated and embedded in Spurr resin (Spurr, 1969) and sectioned 2 µm thick using the Reichert 'Om U 2' Ultramicrotome (Reicher, Vienna, Austria) and stained with 0.5 % toluidine blue in 0.1% sodium carbonate (pH 11.1).

Two regions of mesocarp tissue (cells proximate to the endocarp and to the exocarp), ten cells per region, were selected per cross section avoiding vascular bundles. Three cross sections per fruit, four fruit per treatment, were viewed through a compound microscope at 10X magnification. The image was relayed through a video camera (Color Video Camera/CCD-IRIS, Sony, Japan) to an attached Magnavox computer monitor, and the size of mesocarp cells was determined using image analysis software (Northern Exposure, Empix Imaging Inc., Mississauga, Canada). Statistical analysis was completed by Roisin McGarry, University of Alberta, using The General Linear Model of SAS 6.10 Program (SAS Institute Inc, Cary, USA) following a completely randomized design.

RESULTS

GA_3- and 4-Cl-IAA-stimulated Pericarp Growth

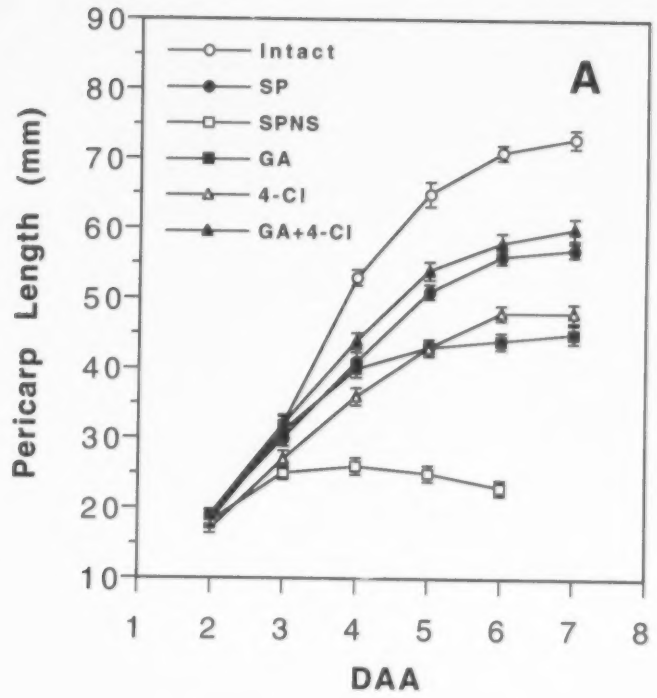
Splitting of the pericarp 2 DAA without disturbing the seeds (SP) reduced pericarp growth compared to the intact pericarp by 18% at 7 DAA (Fig. 5.1). Removal of the seeds (SPNS) at 2 DAA resulted in slowing of pericarp growth and subsequent abscission. Treatment with GA_3 or 4-Cl-IAA stimulated growth of deseeded pericarps compared to the deseeded control (SPNS). Application of GA_3 plus 4-Cl-IAA had additive effects on growth of deseeded pericarps, resulting in growth similar to that of pericarp with seeds (SP).

Histone H2A and γ–TIP expression

Histone H2A and γ–TIP gene expression were investigated during flowering and early fruit development in pea pericarp by RNA gel blot analysis (Fig. 5. 2 and 5.3). The expression of histone H2A in the pericarp was the highest from -2 to 2 DAA and then declined rapidly (Fig. 5.2 and 5.3A). At 5 DAA, H2A transcript levels were only 5% of the original levels at 2 DAA. Expression of γ–TIP was low during the early stages of fruit development (-2 to 1 DAA; Fig. 5.2 and 5.3B), increased after 1 DAA, reached maximum levels at 3 to 4 DAA, and then decreased to levels similar to pre-pollinated fruit (-2 DAA) at 7 DAA. The percent relative abundance of γ–TIP mRNA paralleled the rate of pericarp elongation (Fig. 5.3B).

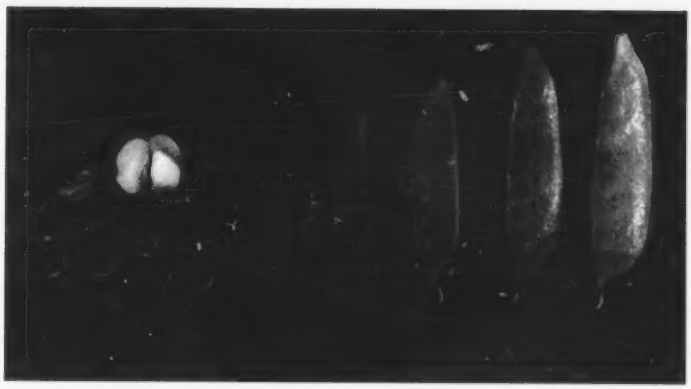
SP treatment exhibited a similar pattern of H2A expression compared to the intact treatment (2 to 7 DAA; Fig. 5.2 and 5.4). Seed removal accelerated the decline in H2A mRNA levels after 3 DAA (Fig. 5.4). In general, deseeded pericarp treated with GA_3, 4-Cl-IAA or GA_3 plus 4-Cl-IAA maintained H2A mRNA levels similar to levels detected in pericarp with seeds (Fig. 5.4) with one exception. At 7 DAA, all hormone-treated deseeded pericarps exhibited higher H2A mRNA levels than pericarps with seeds.

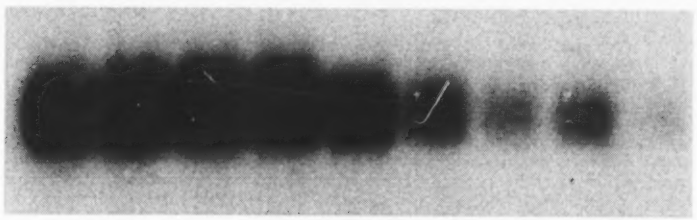
Figure 5.1. The effect of pericarp splitting (SP), seed removal (SPNS), GA_3 (GA), 4-Cl-IAA (4-Cl), and GA_3 plus 4-Cl-IAA (GA+4-Cl) on pea pericarp growth (A). Pericarps at 2 DAA were split (SP) or split and deseeded. GA_3 and/or 4-Cl-IAA or 0.1% Tween 80 were applied immediately after deseeding. Hormones (50 μM) were applied daily for 5 days (total 180 μL). Data are means ± SE (n = 5). Representative pericarps harvested at 7 DAA are shown in picture (B).

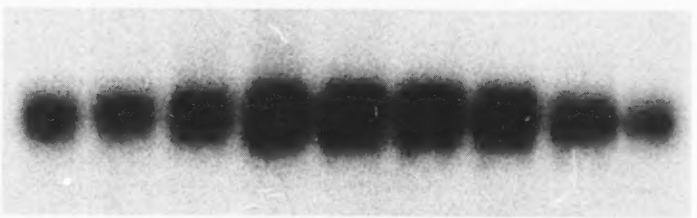

Intact
SP
SPNS
GA
4-Cl
GA+4-Cl
A
Pericarp Length (mm)
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
DAA

B
Intact
SP
GA
4-Cl
GA+4-Cl

Figure 5.2. The development of early pea fruit from -2 to 7 DAA and the corresponding mRNA profiles of histone H2A-1 and γ–TIP in the pericarp tissue. Flower bud and flower shown in picture are at -2 and 0 DAA, respectively.




H2A
0.6 kb


γ-TIP
1.4 kb

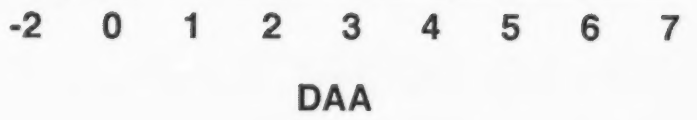
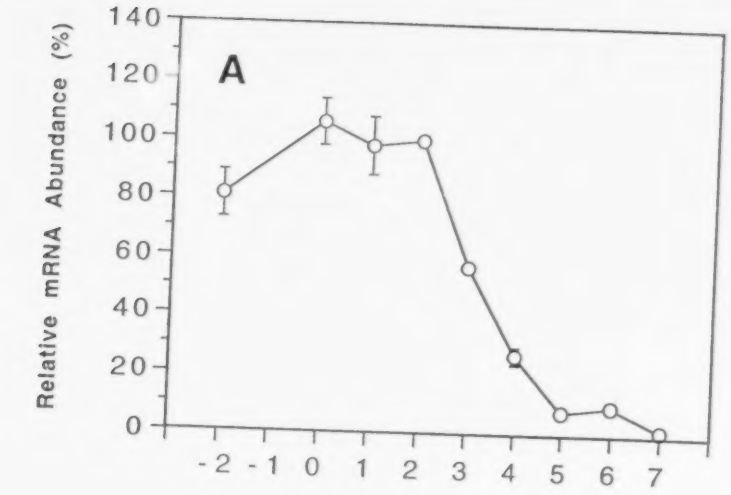

-2 0 1 2 3 4 5 6 7
DAA

Figure 5.3. Relative abundance of histone H2A (A) and γ–TIP mRNA (B) from -2 to 7 DAA in pea pericarp and the corresponding growth rate of pea pericarp (length). Hybridization signals were analyzed by scanning autoradiograms with an imaging densitometer and normalized to the value for pericarps at 2 DAA. The data represents the average of two replicates ± SE (n = 2), growth rate data are means ± SE (n = 5).


A
Relative mRNA Abundance (%)
140
120
100
80
60
40
20
0
-2 -1 0 1 2 3 4 5 6 7

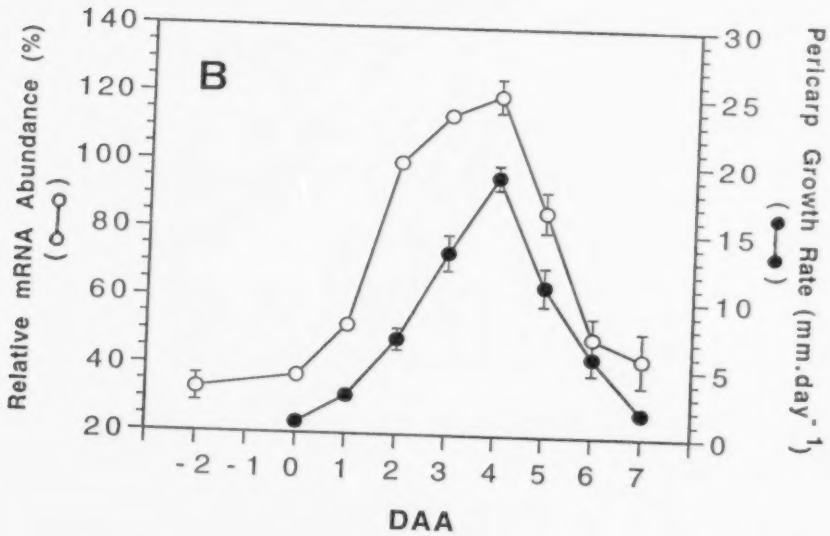
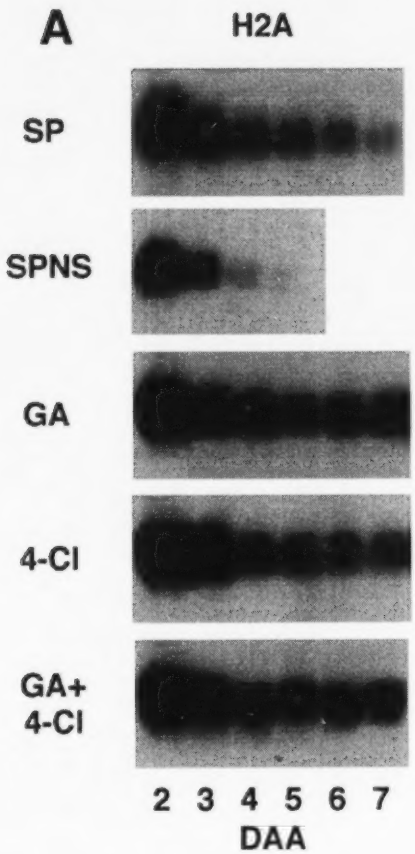
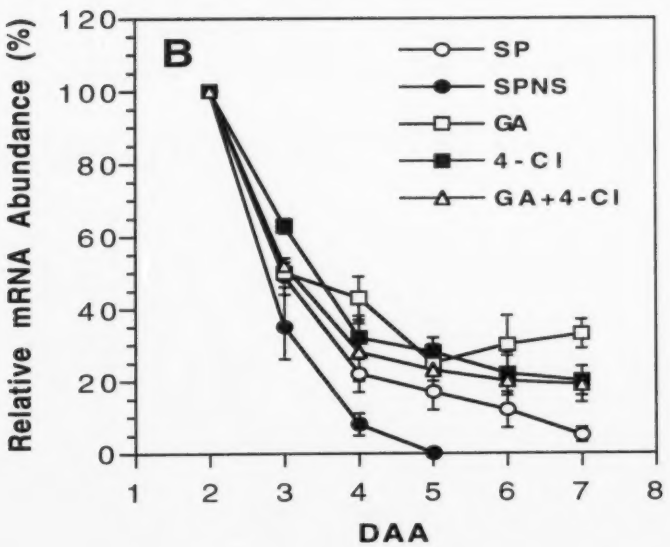

B
Relative mRNA Abundance (%)
Pericarp Growth Rate (mm.day-1)
140
120
100
80
60
40
20
30
25
20
15
10
5
0
-2 -1 0 1 2 3 4 5 6 7
DAA

Figure 5.4. RNA gel blot analysis of histone H2A in pericarp with seeds (SP), deseeded pericarp (SPNS), and deseeded pericarp treated with GA_3 (GA), 4-Cl-IAA (4-Cl), and GA_3 plus 4-Cl-IAA (GA+4-Cl) from 2 to 7 DAA. Pericarps at 2 DAA were split (SP) or split and deseeded. The initial hormone treatments were applied immediately after deseeding and daily thereafter (50 µM; 30 µL at 2 and 3 DAA; 40 µL at 4, 5, and 6 DAA). Pericarps were harvested at the indicated times 24 h after hormone treatment. Profiles of H2A gene expression (A), and percent relative abundance of H2A mRNA levels (B). Hybridization signals were analyzed by scanning autoradiograms with an imaging densitometer and normalized to the value for pericarps at 2 DAA. For the GA_3 (GA) and 4-Cl-IAA (4-Cl) treatments, a 2 DAA sample was not run simultaneous with other samples (3-7 DAA). A representative 2 DAA sample was inserted in the photo for visual comparison. Data are means ± SE (n = 2)


A
H2A
SP
SPNS
GA
4-Cl
GA+
4-Cl
2 3 4 5 6 7
DAA


B
SP
SPNS
GA
4-Cl
GA+4-Cl
Relative mRNA Abundance (%)
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8
DAA

Pericarp γ–TIP mRNA levels in the SP treatment were similar to levels in the intact treatment (2 to 7 DAA; Fig. 5. 2 and 5. 5). Seed removal (SPNS) dramatically decreased γ–TIP expression compared to SP after 3 DAA (Fig. 5.5). γ–TIP transcript levels in 4-Cl-IAA-treated pericarp tissue were similar to levels in the SP throughout the entire experiment (3- 7 DAA; Fig 5.5). γ–TIP mRNA levels in GA_3-treated deseeded pericarp were similar to SP from 3 to 5 DAA. The highest γ–TIP expression in pericarp tissue was observed in the GA_3 plus 4-Cl-IAA treatments at 3 DAA. In contrast to the SP and 4-Cl-IAA-treated deseeded pericarp, all GA_3-treated deseeded pericarps maintained elevated γ–TIP mRNA levels in the later stages of pericarp development (GA_3, 6 and 7 DAA; GA_3 plus 4-Cl-IAA, 5 to 7 DAA; Fig. 5.5).

To evaluate whether the increase in γ–TIP mRNA levels was a function of direct GA induction or more generally associated with the cell enlargement processes, pericarps (2 DAA) were treated with a GA biosynthesis inhibitor (paclobutrazol) to reduce GA levels to a minimum. Twenty four h after the paclobutrazol application, pericarps were treated with 4-Cl-IAA to stimulate pericarp growth. Both GA_3 and the paclobutrazol plus 4-Cl-IAA treatments maintained γ–TIP expression and pericarp growth to a similar extent.

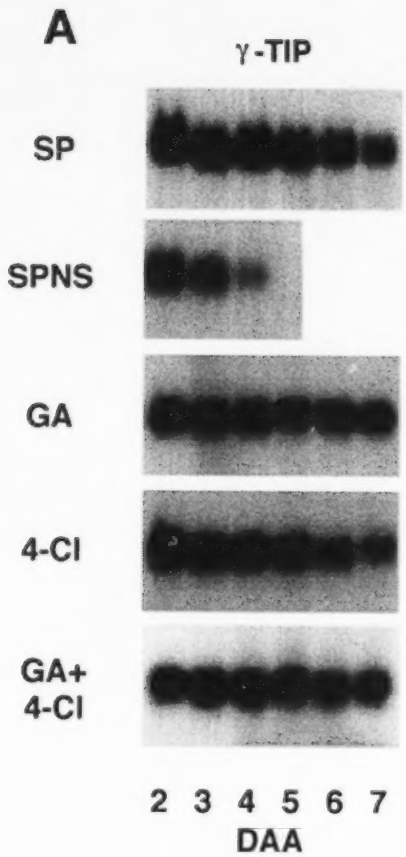
Structural Studies of hormone-treated pea pericarp

To attribute biological relevance to the expression of H2A and γ–TIP genes and to investigate the structural changes associated with GA_3 and/or 4-Cl-IAA induced pericarp growth, histological studies were undertaken. The pea pericarp consists of three distinct tissue layers: exocarp, mesocarp, and endocarp. The exocarp is comprised of a uniseriate epidermis, the mesocarp is composed of approximately 15 layers of vacuolated parenchyma cells, and the endocarp is composed of several layers of small undifferentiated cells. Rapid cellular expansion (30 fold increase in pericarp wall thickness) occurred within the

mesocarp of the intact fruits from 0 to 7 DAA (Fig. 5.7 and 5.9). During this period of fruit development, progressive proliferation and differentiation in the endocarp and vascular bundles were observed (Fig. 5.7). At 0 DAA the endocarp consists of three layers: the inner epidermis, the middle zone (2 layers thick), and a transition layer lining the mesocarp. At 2 DAA the middle zone has 4 layers of cells. Between 2 and 7 DAA, the middle zone differentiated into 5-6 layers of parenchyma cells and 2-3 layers of sclerenchyma cells. At 7 DAA the endocarp is composed of 4 distinct layers: an inner epidermis, a midregion of 5-6 layers of thin walled parenchyma, an inner layer of sclerenchyma, 2-3 cells thick, and a transition layer lining the mesocarp (Fig. 5.7C). Differentiation of the endocarp in deseeded pericarp treated with GA_3 and/or 4-Cl-IAA at 7 DAA was similar to pericarp with seeds (Fig. 5.8).

When 2 DAA deseeded pericarps were treated with GA_3 and/or 4-Cl-IAA, enlargement of the mesocarp cells was observed five days after initial treatment (7 DAA; Fig. 5.8 and 5.9). SP and GA_3 plus 4-Cl-IAA-treated deseeded pericarps had significantly larger mesocarp cells than pericarps treated with GA_3 or 4-Cl-IAA only (LSD, $P<0.05$). The size of mesocarp cells proximate to the endocarp was significantly larger in GA_3- and GA_3 plus 4-Cl-IAA-treated deseeded pericarps (LSD < 0.05). The most pronounced differences between these regions was observed for the GA_3-treated tissue (cells proximate to endocarp were a 1.1 and 3.3 fold larger than cells proximate to the exocarp for GA_3 plus 4-Cl-IAA and GA_3 treatments, respectively). The mesocarp cell size was more homogenous within the 4-Cl-IAA treatment. To determine if the observed heterogenity in mesocarp cell size in GA_3-treated pericarp was an application effect, GA_3 was applied to the exocarp of the pericarp. Application of GA_3 to the exocarp also resulted in larger mesocarp cells in the layers proximal to the endocarp (data not shown). Substantial trichome development from the inner epidermis of the endocarp was observed in pericarp with seeds (SP) and the hormone-treated deseeded pericarps (Fig. 5.8).

Figure 5.5. RNA gel blot analysis of γ–TIP in pericarp with seeds (SP), deseeded pericarp (SPNS), and deseeded pericarp treated with GA_3 (GA), 4-Cl-IAA (4-Cl), and GA_3 pius 4-Cl-IAA (GA+4-Cl) from 2 to 7 DAA. Pericarp treatment, harvesting, and quantitation of mRNA levels were performed as describe in Fig. 5.4. Profiles of γ–TIP gene expression (A), and percent relative abundance of γ–TIP mRNA levels (B). Data are means ± SE (n = 2)


A
γ-TIP
SP
SPNS
GA
4-Cl
GA+
4-Cl
2 3 4 5 6 7
DAA

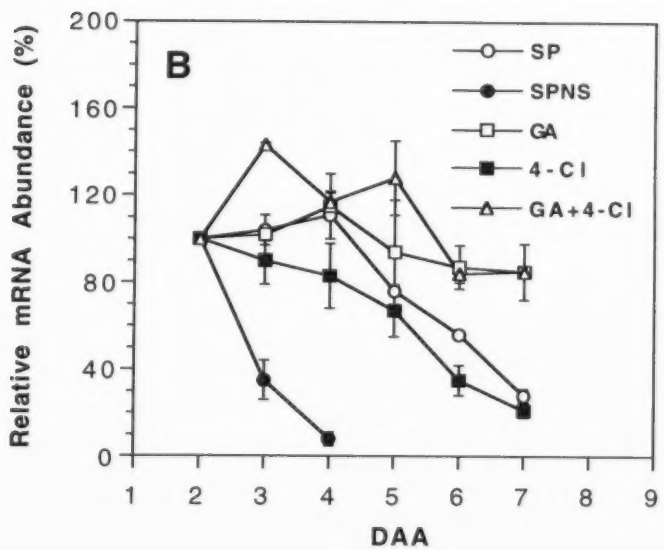

B
SP
SPNS
GA
4-Cl
GA+4-Cl
Relative mRNA Abundance (%)
200
160
120
80
40
0
1 2 3 4 5 6 7 8 9
DAA

Figure 5.6. RNA gel blot analysis of γ-TIP in pericarp with seeds (SP), deseeded pericarp (SPNS), and deseeded pericarp treated with paclobutrazol plus 4-Cl-IAA (Pac+4-Cl), or GA_3 (GA) (A); percent relative abundance of mRNA (B), and the corresponding pericarp growth (C). Pericarps at 2 DAA were split (SP) or split and deseeded. In one treatment, paclobutrazol (30 μL) was applied immediately to the pericarps after deseeding. 4-Cl-IAA (30 μL) and GA_3 (30 μL) were applied 24 h after deseeding; the arrow indicates the time of hormone application. Pericarps were harvested for RNA extraction 24 h after hormone application. Lanes 1-7 on the RNA blot (A) correspond to treatments 1-7 on graph B. Quantitation of mRNA levels were performed as describe in Fig. 5.4. Northern blot analysis data are means ± SE (n = 2), growth rate data are means ± SE (n = 6).

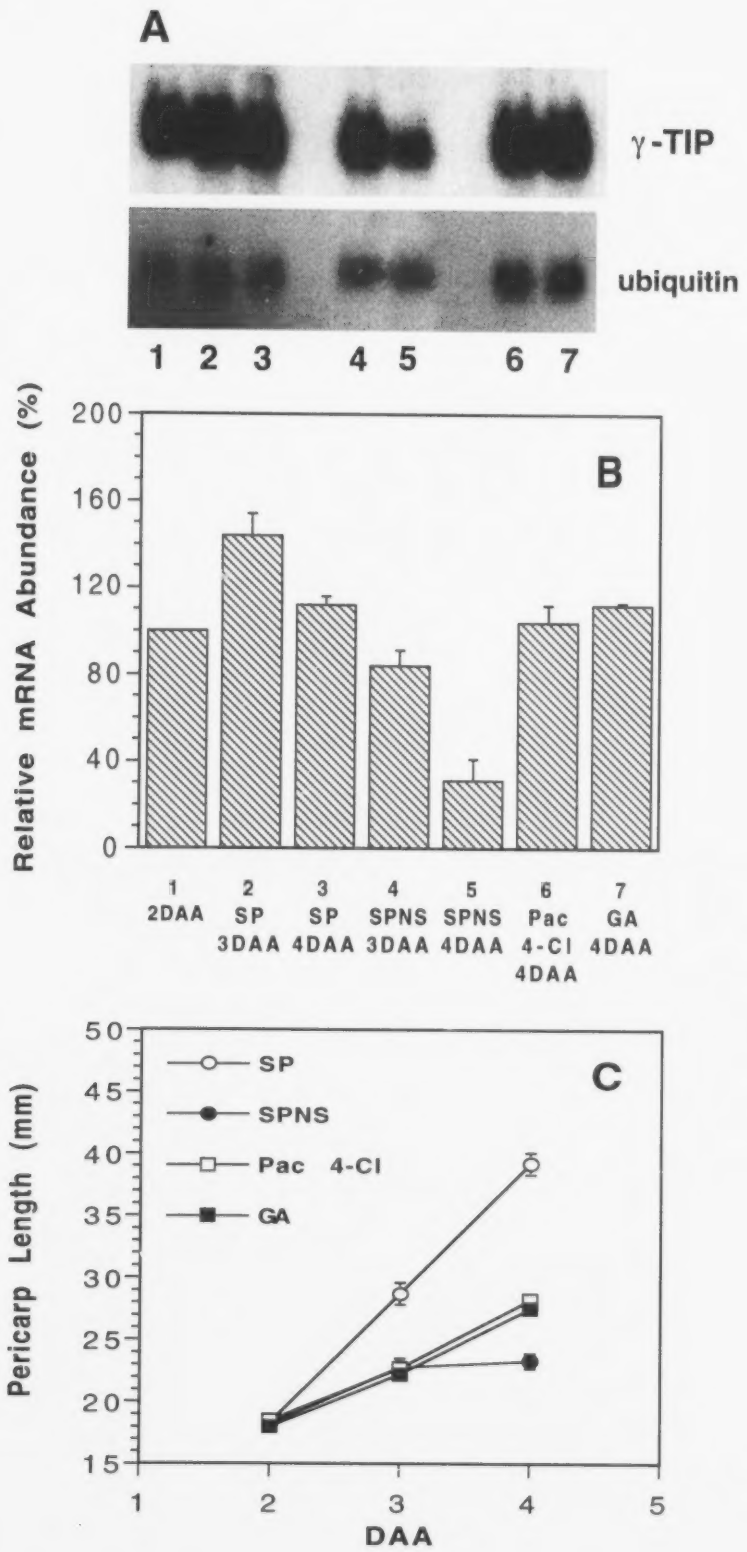

A
γ-TIP
ubiquitin
1 2 3 4 5 6 7
B
Relative mRNA Abundance (%)
200
160
120
80
40
0
1 2DAA
2 SP 3DAA
3 SP 4DAA
4 SPNS 3DAA
5 SPNS 4DAA
6 Pac 4-Cl 4DAA
7 GA 4DAA
C
Pericarp Length (mm)
50
45
40
35
30
25
20
15
SP
SPNS
Pac 4-Cl
GA
1
2
3
4
5
DAA

Figure 5.7. Light micrographs of transverse sections of the mid-region of intact pea pericarp at 0 DAA (A), 2 DAA (B), and 7 DAA (C). Sections were 2 µm thick and stained with Toluidine Blue. IE, inner epidermis; P, middle zone parenchyma; S, sclerenchyma layer; TL, transition layer; V, vascular bundles.

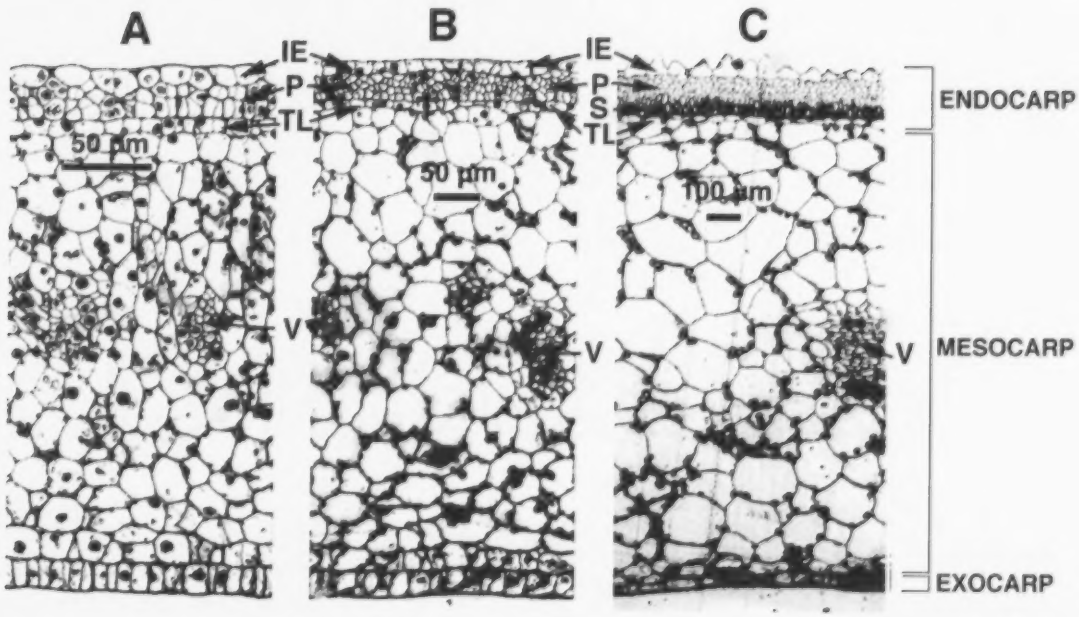

A
B
C
IE
P
TL
S
V
50 µm
100 µm
ENDOCARP
MESOCARP
EXOCARP

Figure 5.8. Light micrographs of transverse sections of the mid-region of pea pericarp with seeds or deseeded pericarp treated with hormones. Split pericarp (SP, A), deseeded pericarp treated with GA_3 (B), 4-Cl-IAA (C), and GA_3 plus 4-Cl-IAA (D). Pericarps were treated as described in Fig. 5.1, and harvested at 7 DAA). Sections are 2 μm thick and stained with Toluidine Blue.

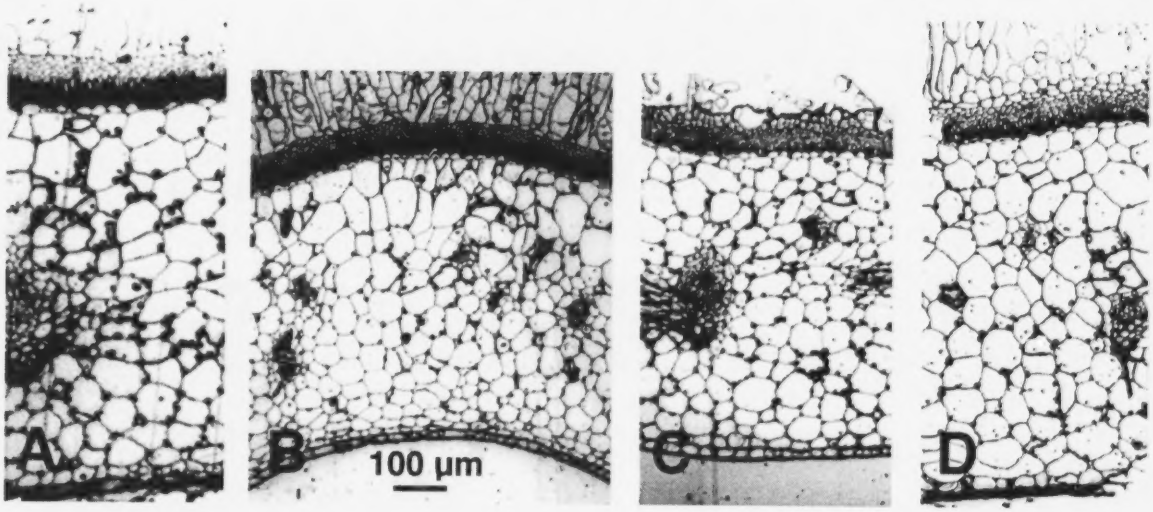
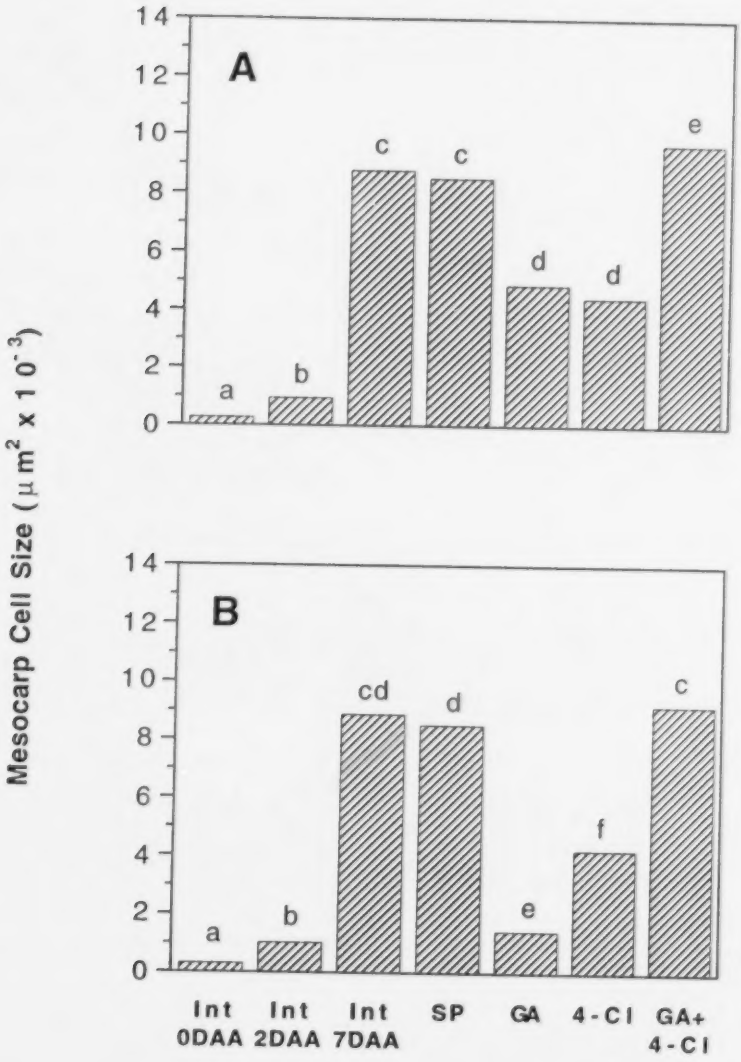

A
B
100 µm
C
D

Figure 5.9. Effect of pericarp splitting (SP), seed removal (SPNS), GA_3 (GA), 4-Cl-IAA (4-Cl), and GA_3 plus 4-Cl-IAA (GA+4-Cl) on mesocarp cell size in pea pericarp. Pericarps were treated as described in Fig. 5.1 and harvested at 7 DAA. Int 0, Int 2, and Int 7 DAA are intact pericarps at 0, 2, and 7 DAA, respectively. Mesocarp cell size proximate to the endocarp (A) and mesocarp cell size proximate to the exocarp (B). Mean separation among treatments within mesocarp regions (a-f) by LSD; $P < 0.05$.


A
B
Mesocarp Cell Size (μ m² x 1 0⁻³)
14
12
10
8
6
4
2
0
a
b
c
c
d
d
e
cd
d
e
f
c
Int 0DAA
Int 2DAA
Int 7DAA
SP
GA
4-Cl
GA+ 4-Cl

DISCUSSION

To gain a better understanding of the cell division and elongation processes during early pea fruit development, we studied the expression of histone H2A and γ–TIP genes in intact pericarp tissue from pre-pollination (-2 DAA), through the period of rapid pericarp elongation, to 7 DAA. The expression of the γ–TIP and H2A genes in pea pericarp was found to be developmentally regulated. Our results showed that the expression of γ–TIP paralleled the rate of pericarp elongation (Fig. 5.3). Our histological studies confirm previous work (Vercher et al, 1984) that the increase in pea pericarp size is due mainly to enlargement of the mesocarp, which constitutes, at 7 DAA, approximately 80 to 90% of the pericarp volume (Fig. 5.7). Also in agreement with observations of Vercher et al. (1984) using -2 to 4 DAA pea ovaries, we observed little or no cellular division in the mesocarp between 0 and 7 DAA, therefore enlargement of the mesocarp during this time is primarily due to an increase in cell size. Cellular divisions were high in vascular tissues and the middle zone parenchyma cells of the endocarp at 0 and 2 DAA (Fig. 5.7). These anatomical observations are consistent with those reported in pea pericarp by Vercher et al. (1984). Since these histological studies indicate that at 0 and 2 DAA the endocarp and the vascular tissues are the major zones in which cell division occurs, it is likely that the high histone H2A gene expression during this period is specific to these tissues within the pericarp. The observed expression patterns of histone H2A and γ–TIP genes suggest that the cell division and cell elongation phases overlap in early pea pericarp development (Fig. 5.2 and 5.3), with the majority of the cell enlargement likely occurring in the mesocarp and the cell division in the endocarp and vascular tissues (Fig. 5.7).

Using the split-pericarp system, we studied the effect of seeds and hormones on growth and development of the pea pericarp. Removal of seeds caused a rapid decrease in growth (Fig. 5.1) and a reduction in abundance of the histone H2A and γ–TIP mRNA levels (Fig. 5.4 and 5.5) in the pericarp. Treatment of deseeded pericarp at 2 DAA with 4-

Cl-IAA and/or GA_3 stimulated pericarp growth and maintained H2A gene expression similar to pericarp with seeds (Fig. 5.1 and 5.4). γ-TIP gene expression in 4-Cl-IAA-treated deseeded pericarp was similar to that in pericarp with seeds (Fig. 5.4). These results suggest these hormones play an important role in maintaining cell division and enlargement during normal pea fruit development. GAs and auxins have been shown to regulate cell division and cell enlargement in other systems as well (Bayliss, 1985; Sauter and Kende, 1992; Hentrich et al., 1985; Koornneef et al. 1990; Phillips and Huttly, 1994).

Phillips and Huttly (1994) found that γ–TIP mRNA abundance was markedly increased in the *Arabidopsis ga1* mutant after GA application. To test whether the increase in γ-TIP mRNA abundance during pericarp growth is a function of direct GA induction or more generally associated with cell enlargement, paclobutrazol was applied to pericarps (2 DAA) to reduce GA levels to a minimum. Application of 4-Cl-IAA to paclobutrazol-treated pericarp stimulated pericarp growth and maintained γ-TIP expression. These data suggest that γ–TIP gene expression in pea pericarp is associated with the cell elongation process and is not simply a function of endogenous GA levels in the tissue. However, when deseeded pericarps were exposed to exogenous GA_3 at levels which give an optimal growth response (50 μM; GA_3 and GA_3 plus 4-Cl-IAA), it appears that GA_3 is directly effecting the γ–TIP steady state mRNA levels. One possibility is that the daily application of GA_3 increased the stability of γ–TIP transcripts in pea pericarp, which could explain the high γ–TIP mRNA levels at a time when the rate of elongation was low (6 and 7 DAA). In *Arabidopsis*, one application of GA_3 increased the γ–TIP mRNA levels for at least 96 h (Phillips and Huttly, 1994). Further work is required to determine if GA_3 at 50 μM is effecting γ-TIP mRNA levels through transcriptional or post-transcriptional mechanisms.

GA_3 application to deseeded pericarp promoted cell enlargement mainly in cells of the mesocarp proximal to the endocarp, suggesting that stimulation of cell expansion by GA_3 is cell specific within the mesocarp tissue. The spatial responsiveness to GA_3 could

be the consequence of changes in the levels of active GAs within these cells or changes in hormone sensitivity. 4-Cl-IAA application to deseeded pericarp, in contrast, stimulated cell enlargement in all layers of the mesocarp. 4-Cl-IAA application to GA_3-treated deseeded pericarp (GA_3+4-Cl-IAA) had additive effects on mesocarp cell size proximate to the endocarp and synergistic effects on mesocarp cell size proximate to the exocarp (Fig. 5.9). These data suggest that the cells proximate to the exocarp are responsive to GAs in the presence of 4-Cl-IAA. Vercher and Carbonell (1991) did not observe differential cell enlargement in the mesocarp of GA_3 treated unpollinated fruits at 5 DAA. The differences observed between our studies and Vercher and Carbonell's may be may be due to the type of tissue used and the timing of the GA_3 treatments (application of GA_3 to unpollinated ovaries at 0 DAA by Vercher and Carbonell (1991) versus application of GA_3 to deseeded pollinated ovaries at 2 DAA in our studies).

Pea fruit development involves a balance of cell division, elongation, and differentiation. Our histological and histone H2A expression studies indicate that GA_3 and 4-Cl-IAA effects on cell division in post-anthesis pericarp are similar. Therefore, the differences observed in growth between the GA_3 plus 4-Cl-IAA-treated deseeded pericarp and deseeded pericarp treated with GA_3 or 4-Cl-IAA only, are more likely to be a function of cell enlargement rather than cell division. Application of GA_3 plus 4-Cl-IAA had additive and synergistic effects on mesocarp cell size, suggesting an interaction of GA_3 and 4-Cl-IAA on cell enlargement in pea pericarp. The mesocarp cell size in GA_3 plus 4-Cl-IAA-treated deseeded pericarp was similar to that in pericarp with seeds, supporting our hypothesis that both hormones are involved in pea fruit development.

In conclusion , our studies suggest that γ-TIP and histone H2A genes can be used as molecular markers to characterize mitotic activities and cellular expansion during early pea fruit development. The pattern of cell division and enlargement in pea pericarp is disrupted when seeds are removed at 2 DAA, suggesting that sustenance of these processes in pea fruit requires the presence of the seeds. Our northern blot analysis as well as our

histological studies indicate that the differences observed in growth in GA and/or 4-Cl-IAA treatments are more likely to be a function of cell elongation rather than cell division. We also found that exogenous GA treatments to deseeded pericarp resulted in elevated steady state mRNA levels of γ-TIP, which were not necessarily associated with pericarp growth. Therefore, these experiments suggest that γ-TIP is not an appropriate molecular marker for cell enlargement when exogenous GA treatments are used.

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Chapter 6

Summary and Conclusions

Pea fruit is a model system for studying the effect of seeds on fruit development. Fruit set and sustained pericarp elongation in pea are dependent on the presence of seeds. The studies reported herein investigated the biochemical and molecular interplay between developing seeds and the pea pericarp. The effect of seeds on pea pericarp growth is assumed to involve plant hormones, specifically GAs and auxins (4-Cl-IAA). The objective of this research was to examine the relative roles of GA and 4-Cl-IAA and their interaction in young developing fruit. Previous studies using the substrate [^{14}C]GA_{12} suggested that seeds stimulate a key step in the GA biosynthetic pathway (conversion of GA_{19} to GA_{20}; Ozga et al., 1992). Preliminary experiments showed that 4-Cl-IAA can substitute for the seeds in the conversion of putative GA_{19} to GA_{20} (Ozga and Brenner, 1992). This research specifically tested the hypothesis that auxin has two regulatory roles in controlling fruit growth: 1) the export of 4-Cl-IAA from the seeds to the pericarp where it stimulates GA biosynthesis; 2) a direct auxin effect of 4-Cl-IAA on pericarp growth. [^{14}C]GA_{19} metabolism was studied in pea pericarp to investigate further seed and 4-Cl-IAA regulation of GA biosynthesis in pericarp tissue, specifically, conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20}. The results of this study showed that [^{14}C]GA_{19} is metabolized to [^{14}C]GA_{20} by pea pericarp when seeds are present. However, when seeds are removed, the conversion [^{14}C]GA_{19} to [^{14}C]GA_{20} was greatly reduced or inhibited. Our data also showed that 4-Cl-IAA could stimulate the conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20} in deseeded pea pericarp. Although the presence of 4-Cl-IAA in pea seeds has been known since the late 1960s (Marumo et al., 1968), no specific function for this halogenated auxin has been found. Endogenous 4-Cl-IAA may play a specific role as a seed signal by stimulating GA biosynthesis in the pericarp.

The data suggesting that seed and 4-Cl-IAA may regulate the conversion of GA_{19} to GA_{20} in the GA biosynthesis pathway led us to examine the role of GA 20-oxidase in this regulation. GA 20-oxidase, a multifunctional enzyme, has been shown to catalyzes the biosynthetic sequences GA_{53} -> GA_{44} -> GA_{19} -> GA_{20} and GA_{19} -> GA_{17} (Lange et al., 1994). GA 20-oxidase expression was investigated in pericarp with seeds and hormone-treated deseeded pericarp by RNA gel blot analysis. Our results showed that GA 20-oxidase transcript levels are maintained in pea pericarp when seeds are present. In contrast, seed removal reduced GA 20-oxidase mRNA levels dramatically. Application of 4-Cl-IAA to deseeded pericarp increased GA 20-oxidase mRNA transcript levels.

Both our [^{14}C]GA_{19} metabolism data and the GA 20-oxidase expression studies support the hypothesis that seeds maintain GA biosynthesis in pea pericarp and that 4-Cl-IAA, a putative transmittable seed factor, may be responsible for stimulating GA biosynthesis in the pericarp, specifically the conversion of GA_{19} to GA_{20}. Our results suggest that the conversion of GA_{19} to GA_{20} may be controlled, at least in part, through regulation of the GA 20-oxidase transcript levels.

A decrease in GA 20-oxidase mRNA levels was observed in GA_3 treated deseeded pericarp, supporting the view that bioactive GAs may control their own synthesis through down regulation of GA 20-oxidase gene expression. Further research will be required to examine whether 4-Cl-IAA and GA are regulating the rate of transcription and/or the stability of GA 20-oxidase transcripts.

The identification and isolation of other cloned GA biosynthetic genes is important to further explore all aspects of GA biosynthetic gene regulation in pea fruit development. Our [^{14}C]GA_{19} metabolism studies suggest that the 2ß-hydroxylation of GA_{20} to GA_{29} may also be seed regulated. A higher ratio of [^{14}C]GA_{29} to [^{14}C]GA_{20} was observed in the SPNS control and the 4-Cl-IAA-treated deseeded pericarp than in pericarp with seeds. It would be interesting to study the expression of 2ß-hydroxylation genes in pericarp with

and without seeds, however so far 2ß-hydroxylation genes have not been cloned. Since the cloning of GA 20-oxidase by Lange et al., (1994), *ent*-kaurene synthase genes of *Arabidopsis* (Sun and Kamiya, 1994), maize (Bensen et al., 1995), pea (Ait-Ali et al., 1996), pumpkin (Yamaguchi et al., 1996), and a putative GA 3ß-hydroxylase of *Arabidopsis* (Chiang et al., 1995) have been cloned. The availability of these *ent*-kaurene synthase, GA 20-oxidase, and GA 3ß-hydroxylase genes will enhance our understanding of how GA biosynthesis in pea is regulated in response to both endogenous and environmental signals.

To assess at the protein level the relative contribution of GA_3 and 4-Cl-IAA to pea fruit development, *in vivo* labeling with [^{35}S]methionine coupled with two-dimensional gel electrophoreseis was used to characterize de novo synthesis of proteins during GA_3, 4-Cl-IAA, and seed-induced pea pericarp growth. The polypeptide changes observed could be grouped into 6 classes: 4-Cl-IAA-induced, 4-Cl-IAA-repressed, GA_3-induced, GA_3- and 4-Cl-IAA induced, GA_3- and 4-Cl-IAA-repressed, and seed-induced. The greatest difference in *in vivo* labeled polypeptide patt(.is occurred between pericarp with seeds and deseeded pericarp. Seed removal caused the pea pericarp to synthesize, inhibit or modify specific proteins. Application of 4-Cl-IAA and GA_3 to pericarp 12 h after deseeding reversed this process. GA and auxin have the ability to induce and repress specific proteins in other plant systems as well (Theologis, 1986; Chory et al., 1987). Similar patterns of protein expression were associated with both hormone treatments; however, polypeptide patterns unique to GA_3 or 4-Cl-IAA also indicated that GA_3 and 4-Cl-IAA effects are not equivalent. In general, the results demonstrated that application of 4-Cl-IAA plus GA_3 replaced the seed effect on protein synthesis.

The final objective was to examine further the relative roles of auxins and GAs on cell division and cell expansion during pea fruit development. Using RNA gel blot analysis, histone H2A and γ-TIP gene expression were monitored as markers for cell

division and cell elongation, respectively. The expression of the H2A and γ-TIP genes were found to be developmentally regulated. Our studies suggests that γ-TIP and histone H2A genes can be used as molecular markers to characterize mitotic activities and cellular expansion during early pea fruit development. The northern blot analysis as well as the histological studies indicate that the differences observed in growth in GA_3 and/or 4-Cl-IAA treatments are more likely to be a function of cell enlargement rather than cell division.

The pea fruit is a useful model system in understanding the roles of GAs and auxins in fruit development. Our [^{14}C]-GA_{19} metabolic and the molecular studies suggest that the conversion of GA_{19} to GA_{20}, is controlled by seeds and 4-Cl-IAA, at least in part through regulating GA 20-oxidase transcript levels. These data support our hypothesis that 4-Cl-IAA may be a seed-derived signal stimulating GA biosynthesis in the pea pericarp. Thus far, few studies have shown that one plant hormone can specifically affect the biosynthesis of another hormone (Botella et al., 1992; Kim et al., 1992). The *in vivo* protein synthesis and histological studies illustrate that 4-Cl-IAA has auxin effects on growth independent of its effect on GA biosynthesis. These results support the hypothesis that 4-Cl-IAA directly stimulates pericarp development. Using the pea split-pericarp growth system has given us more insight into the relative contributions of GAs and 4-Cl-IAA to fruit development and will enable us to examine further the interaction of GAs and auxins in pea pericarp growth.

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APPENDIX I: Two-Dimensional Gel Electrophoresis

Protein extraction:

1. Homogenize pericarp (0.5 g) to a fine powder with liquid N_2 to a fine powder
2. Add 0.5 mL of extraction buffer (50 mM Tris HCl, pH 7.5, 2% SDS, 150 mM DTT and 1 mM PMSF).
3. Centrifuge extract at 10,000*g* for 10 min
4. Heat clear supernatant for 3 min at 100°C and cool on ice
5. Add 8 volumes of acetone to extract and precipitate proteins at -20°C for at least 1 h
6. Centrifuge at 10,000*g* for 10 at 4°C
7. Remove acetone, air dry pellet, and resuspend pellet in buffer A

Isoelectric Focusing:

Pour tube gels:

1. Fire polish bottom ends of about 18 x 200 µL capillary micropipettes
2. Place capillary pipettes into a 10 mL graduated cylinder with screen at bottom
3. Make gel mixture.

5.5 g	Urea
0.16 g	Chaps
40 µL	Triton-100
1.25 mL	Acryl./Bis. (30%/0.8%) gel stock
200 µL	Ampholytes 4-5
200 µL	Ampholytes 5-7
100 µL	Ampholytes 3-10
fill up with H_2O to 10 mL	

dissolve above components at 37°C, then:

mix and degas,

Add: 10 µL TEMED

20 µL 10% ammoniumpersulfate

Pour into tube-casting graduated cylinder. Fill capillaries to just over black line.

Cover with Saran Wrap and polymerize for 1 h

Make Tank and Buffer Solutions:

Sample Buffer A:

		5 mL
9.5 M	Urea	2.85 g
5 mM	K_2CO_3	0.5 mL from 50 mM stock
0.5%	DTT	50 mg
H_2O		1.6 mL

dissolve at 37°C, then add:

CHAPS	0.4 g
H_2O	0.5 mL
Ampholytes 4-5	100 µL
Ampholytes 5-7	100 µL
Ampholytes 3-10	50 µL

Ampholytes equal 5% of volume at 5 mL

Aliquot, label and store at -70°C

Upper Tank Buffer (0.02 M NaOH): must degas for at least 1 h

1 M NaOH stock	15 mL
H_2O	735 mL

Lower Tank Buffer (0.01 M Phosphoric Acid)

Make 1 N stock solution by adding 23 mL concentrated phosphoric acid (44 N) to 977 mL H_2O

Working solution:

1 N Phosphoric Acid stock	44 mL
H_2O	956 mL

Set up Tube Gel Apparatus:

1. Clean tubes of excess polymerized gel
2. Place tubes in tube gel apparatus
3. Fill tubes with NaOH top chamber buffer. Check for bubbles in tubes
4. Pour enough phosphoric acid bottom chamber buffer into chamber to contact bottom of tubes
5. Assemble top chamber onto bottom chamber.
6. Add samples
7. Top each tube off with NaOH upper tank buffer and fill top chamber with NaOH upper tank buffer
8. Run gel: 2 h at 200 V
   5 h at 500 V
   16 h at 800 V
9. Turn off power, disassemble gel apparatus, place tube gels into microcentrifuge tubes containing sample buffer and store at -70°C or use directly for SDS-PAGE

Sample Buffer:	<u>10 mL</u>
50 mM Tris pH 7.5	1 mL of 1 M Tris pH 7.5 stock
2% SDS	2 mL of 10% SDS stock

10% DTT	1 g DTT
10% glycerol	1 mL glycerol
1% Bromophenol blue	0.1 g Bromophenol blue